THE DEVELOPMENT OF COMPOUNDS FOR THERAPEUTIC USE:

A STUDY OF FACTORS WHICH MODIFY ACTIVITY.

A THESIS

Presented by

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My sincere thanks are also due to all of my staff, in particular to Mr. P. Acred, who has given me such loyal support.

CONTENTS

SECTION B - PHARMACOLOGICAL PAPERS

INTRODUCTION

The papers submitted in this thesis represent various aspects of research in which I have been engaged since 1953, as Head of the Department of Pharmacology and Chemotherapy of Beecham Research Laboratories, Ltd., the Pharmaceutical division of Beecham Group Ltd.

During this time I have had the opportunity to conduct investigative work in two main fields, as indicated by the grouping of the papers. The first deals with the pharmacology of drugs which possess chemotherapeutic activity (Section A); the second, with drugs which act on the autonomic nervous system or modify the inflammatory reaction or act in other ways (Section B).

From the beginning, my object has been to develop methods both in vitro and vivo for the selection of compounds which might be useful in therapeutics. These were then studied extensively in animals so as to establish patterns for their absorption, distribution and excretion, and for their metabolic transformation into active or inactive metabolites. Such information makes it possible to interpret the pharmacological action of a drug and hence

to consider whether or not tests of its activity should be extended to man.

The subject matter of the thesis is of necessity diverse; nevertheless there is an underlying theme, namely the relationship between the chemotherapeutic or pharmacological action of drugs and the manner of their disposal in the body. In the following sections, factors concerned in this disposal are reviewed briefly and references are given to the appropriate texts in the thesis.

ABSORPTION

Absorption of drugs from the intestine is believed to occur by passive diffusion of the unionised molecules and it is frequently assumed that there is little difference in this respect between species, but this is not so. Even in such a series as the semi-synthetic penicillins where differences in physical characteristics such as pk_a and solubility are small, there are distinct variations between species. Furthermore, after parenteral administration of antibiotics in different species the percentage excreted in the urine differs widely. (cf. p. 52 and Knudsen and Rolinson, B.M.J. ii, 1960, pp. 700 - 703). This type of study is only of limited help in extrapolating from animal

to man. Additional factors influencing absorption and blood concentration patterns are the effect of anaesthesia which prolongs the time of absorption (pp.172-175) , or the presence of inflammation which increases both the peak and the duration of blood concentration. Studies in rats have confirmed that, in response to an appropriate inflammatory stimulus, total antibiotic concentrations appearing in the inflammatory fluid are high and may in fact exceed the corresponding concentrations in the blood (pp.157 - 162). Since the assay of tissue concentrations may be unreliable because of residual blood, it is more appropriate to examine concentrations appearing in the peripheral and thoracic lymph. An extensive comparative study in dogs has shown that penicillins penetrate readily into the extravascular fluids whereas sulphadiazine, a typical sulphonamide, does not.
The free concentrations of antibiotics in the lymph correspond to the free concentrations in the blood; hence it is possible to conclude that the activity of the penicillins is not less in the tissues than in the blood.

PROTEIN BINDING

The extent to which a drug is bound by protein largely governs its activity; an estimate of the free concentration is therefore essential in predicting therapeutic value. Initially this was studied by a new method using

Sephadex to separate the free and bound antibiotic and the values agreed closely with those obtained by equilibrium dialysis. (pp.145 - 148). However, the method based on the use of Sephadex turned out to be more difficult than that based on equilibrium dialysis, and it was therefore abandoned in our studies on protein binding. Nevertheless, Sephadex has since been employed in the separation of covalent complexes of penicillin with protein which are partially responsible for the induction of penicillin allergy. (Batchelor, Dewdney, Feinberg & Weston, Lancet, 1967, 1175-1177). Parallel estimations in mice of the degree of protein binding and therapeutic efficacy have shown that these two properties are strongly correlated, i.e. the group of compounds having low binding being more active than compounds with high binding (p. 81). These findings confirmed the in vitro observations that binding leads to a reduction in activity.

REMOVAL FROM THE BODY

Many drugs disappear from the body by excretion in the urine; they may also be removed by the bile and possibly in the breath; minor quantities are excreted in the sweat or in tears. Penicillins are largely excreted in the urine but in addition to glomerular filtration, they are actively

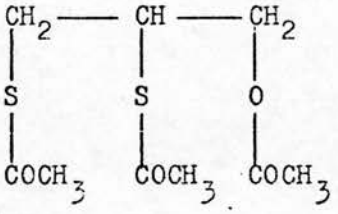
secreted by the renal tubules. This has been demonstrated in the hen by adapting Sperber's technique (Zool. Bidrag Upsalla 1949, 27, 429-488). A compound that is removed from the body by renal tubular secretion is injected into one leg of the hen; the concentration of the compound appearing in the urine is then found to be much greater from the kidney on the side of the injection than from that on the other side. In the hen there is a renal portal system; hence the concentration of antibiotic in blood entering the kidney via the renal portal system on the side of the injection is much greater than in the blood on the contralateral side. After blocking the renal tubular secretion with probenecid the urinary concentrations become equal. These experiments have demonstrated that penicillins are excreted not only by glomerular filtration but also by renal tubular filtration.

Studies in the rat have confirmed that high concentrations of penicillin are also excreted in the bile (p. 110, p.135, p. 180).

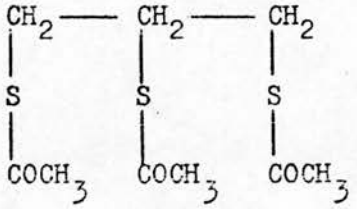
METABOLISM

In a study of antitubercular drugs in mice, it was demonstrated that 2,3-dimercaptopropanol had some slight activity in that it prolongs the survival time. On the basis of this finding a range of thiols, dithiolans, thiol

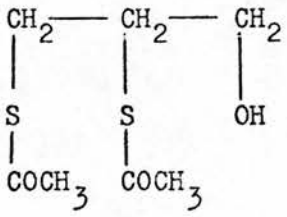
esters, dimercaptopropyl esters and episulphides was examined. The results led to the conclusion that the active moiety was an episulphide and that only compounds that could form episulphides in vitro or in vivo were active (pp.21 - 33). Miles and Owen J. Chem. Soc. 817, 1952 and Hardy and Owen J. Chem. Soc. 1528, 1954, demonstrated that acetylated thiols with a vicinal hydroxy group in the presence of mild alkali are deacetylated with ring closure to form episulphides. The following thiol esters which fulfil these conditions proved to be active.



BRL 403

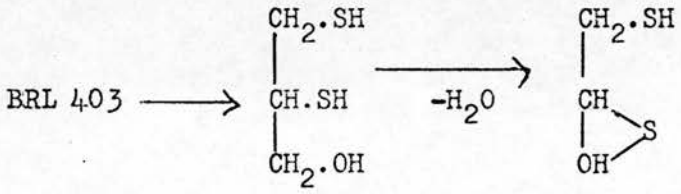


BRL 411

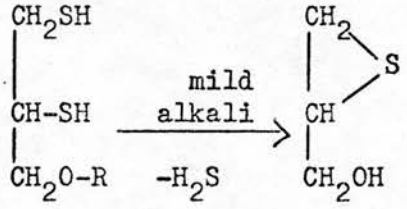


BRL 464

Both in vitro and in vivo there is rapid deacetylation with the elimination of the elements of water to form an episulphide .



Compounds with higher acyl groups do not hydrolyse so readily and hence are less active, and where the potential hydroxy group is separated from the thiol group by one or more carbon atoms the activity is lost. Most esters of dimercaptopropanol were active in vivo. This was due to the hydrolysis of the hydroxy ester, and ring closure then took place to form the corresponding episulphide.

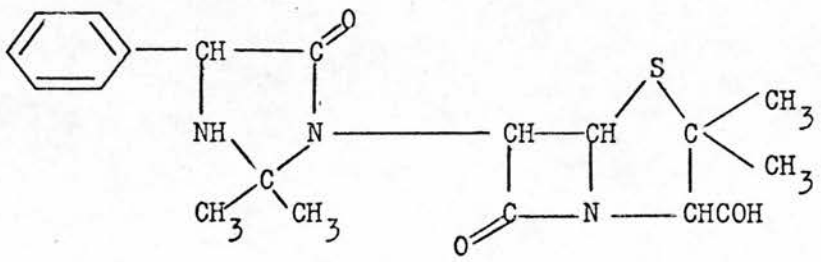


Further support for the metabolic conversion of acetylated thiols to active antitubercular episulphides lies in the observation that 1,3-dimercapto-2-benzoyl propyl ester is highly active both in vivo and in vitro

whereas the parent compound, 1,3-dimercapto-2-propanol (BRL 531), is inactive in vivo and highly toxic.

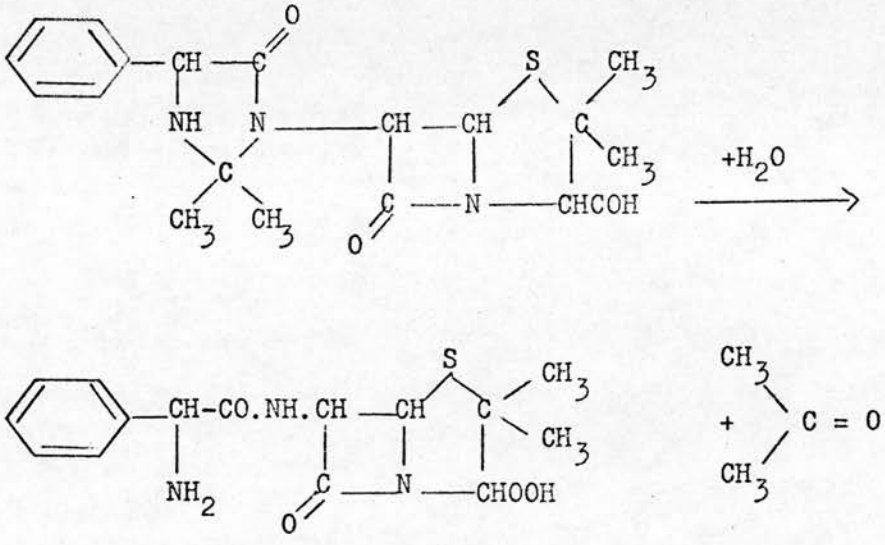
It was possible that the thiols were converted in vivo to ethanethiol since this compound is a very active antitubercular agent. A sample of BRL 587 (Benzyl ester of dimercaptopropanol) labelled with S^{35} was administered subcutaneously to animals and the expired air examined for the presence of radioactivity, but no radioactivity was found. Since ethanethiol is mostly excreted in the breath it could be concluded that no BRL 587 was converted to ethanethiol. However, over 80% of the radioactivity appeared in the urine following the administration of BRL 587, which indicated that the bulk of the compound had been metabolised giving inorganic sulphate.

Another example of conversion to an active compound is that of hetacillin (pp.149-156). Hetacillin, which has the following structure



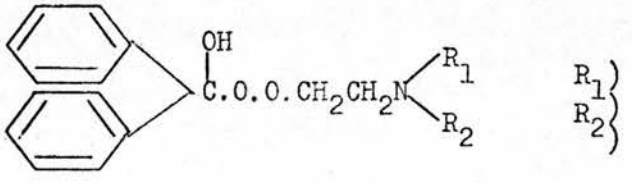
has a spectrum of activity similar to ampicillin. It is metabolised in the body but in this instance the conversion

takes place in the blood. By using high voltage electrophoresis technique it can be demonstrated that hetacillin is converted to ampicillin in the serum,



Hetacillin migrates to the positive pole whereas ampicillin migrates in the opposite direction. Blood determinations in man showed that within ten minutes nearly all of the absorbed hetacillin is converted to ampicillin in the serum.

In a study of basic esters having antiacetylcholine properties, it has been shown in a given series that the central "antiacetylcholine" activity may remain constant whereas the peripheral activity is reduced. Compounds of the following structure were investigated:



Maximal central "antiacetylcholine" activity, measured by its prevention of oxotremorine induced tremors in mice, occurred when R_1 = Et and R_2= Pr^i (BRL 1288), whereas the peripheral antiacetylcholine activity was very low in relation to that of atropine. Metabolic studies (pp.222-244) with the aid of autoradiographic techniques, have shown that this compound is absorbed rapidly from the intestine and is distributed throughout the whole body. Differential assays based on the use of ^{131}I, ^{35}S and ^{14}C-urea indicate that the drug penetrates readily into the brain cells, and thin layer chromatography has confirmed that it is present unchanged. However, in the peripheral circulation within ten to fifteen minutes there is no trace of the parent compound, and eight metabolites have been identified. The metabolism in man is essentially similar to that in rats (p.241). The rapid disappearance from the blood accounts for the low activity in preventing salivation and for its failure to cause mydriasis. This compound has been tested in Parkinson's disease and has been shown to possess marked central activity with little or no antiacetylcholine action. (pp. 218 - 221).

CONVERSION IN THE INTESTINE

An active drug may be destroyed in the intestine, either by the physical conditions within the intestine or by bacteria; alternatively it may be converted to another active compound. An example of this conversion is cloxacillin, an active oral penicillin which is effective against Penicillin G-resistant forms of staphylococci. In the intestine it was converted to a penicillin which no longer displayed activity against these forms of staphylococci (pp.111-112). The conversion is due to an enzyme which is either secreted by the intestine or by bacteria. The compound has still to be identified, but it is probable that the isoxazole ring has been split to produce a compound which can be attacked by penicillinase. (p.16).

CONVERSION TO AN ACTIVE COMPONENT IN THE BODY

Ampicillin when administered to rats gives rise to an active metabolite which appears in the urine. There would appear to be no trace of this compound in the blood and the conversion could take place in the kidneys. Experimental work suggests that this compound has lost its broad spectrum activity, but insufficient work has been done to characterise the particular metabolite.

DRUGS AFFECTED BY ENZYMES

Following the isolation and characterisation of 6-amino-penicillanic acid in our laboratories, the principal objective was to develop a penicillin effective against staphylococci which were resistant to Penicillin G; these had become a serious problem in hospitals. The resistant staphylococci produce a penicillinase which hydrolyses Penicillin G with the production of penicilloic acid. The phenyl group was therefore modified with sterically hindered groups which prevented the access of penicillinase to the molecule. An early penicillin which acted in this way was 2,6-dimethoxybenzyl-penicillin. Before this compound could be given to man it was necessary to show that it was active in vivo. Activity was confirmed by modifying an intitubercular test originally designed by Selby and O'Grady (Brit. J. Exp. Path. 35 556, 1954). Staphylococci sensitive to Penicillin G are injected under the muscle sheath in one hind limb of a mouse and staphylococci resistant to Penicillin G are injected into the other hind limb. The animal is treated with the antibiotic under test and the effectiveness of the therapy is assessed by the reduction of the size of the local lesion produced by the staphylococcus. This

technique therefore provides an excellent model for determining the effect of an antibiotic against two strains of bacteria in the same animal and for demonstrating that penicillinase produced by the resistant staphylococcus is ineffective in destroying the new penicillin (pp. 39 - 44).

The action of drugs can also be modified by enzymatic action within the body. In a series of esters of 1-alkyl-2-hydroxypyrrolidine (pp. 184 - 189) the diamethylaminoethyl ester proved to be the most active antiacetylcholine compound tested in vitro, whereas in vivo its activity relative to others within the series was much less. A method of estimating antiacetylcholine activity was developed in rabbits whereby the flow of saliva was stimulated by carbachol and the effect of the compound was measured by recording the reduction in flow. (pp. 191 - 195). However, the results in different strains of rabbits differed markedly. The reason for this difference was traced to the variability in atropine esterase activity in the serum which hydrolysed the compounds, rendering them inactive. Two factors arose out of this study which previously had not been fully appreciated; firstly atropine esterase activity could vary markedly between rabbit strains - it being absent in one strain: secondly, in a

closely related series of esters the compounds could vary in their stability to atropine esterase. These experiments therefore emphasised the importance of strain variation within a species and the importance of metabolism and factors affecting metabolism in interpreting the action of drugs.

STRAIN DIFFERENCES

The importance of strain difference in pharmacological response was subsequently emphasised in the paper dealing with the variability of mouse strains in the writhing response to phenylquinone (pp.253-259) Inhibition of this response by drugs is taken to indicate mild analgesic activity; a reliable estimate of this activity could not have been obtained if a suitable strain of mouse had not been selected. The difference in response to phenylquinone between the mouse strains has still to be clarified.

TOXICITY

Phenacetin has been widely reported to cause renal papillary necrosis in man, yet studies in animals have yielded little information on how the lesion is produced. An investigation of early kidney damage caused by mild analgesic drugs in animals showed first, that amidopyrine and antipyrene both had a toxic action on the renal tubule; secondly, that amidopyrene also caused renal papillary necrosis; and thirdly

that phenacetin had no effect on either the renal tubule or the papilla. Most of the reports on phenacetin ignore the fact that it is usually compounded with other drugs and this experiment suggested that phenacetin was not the principal agent in causing renal papillary necrosis. Kincaid Smith (B.M.J. 1970, 3, 559) has subsequently confirmed from animal studies that phenacetin is not the only analgesic likely to cause renal papillary necrosis, but that the toxic effects which are usually attributed to phenacetin may be due to other drugs associated with phenacetin in analgesic preparations. In assessing the toxic effects of a drug an animal model may therefore provide useful information in the analysis of the toxicity, and can assist in predicting the adverse reactions when it is given to man.

MISCELLANEOUS PAPERS

Anti-inflammatory test (pp. 278 - 279)

This test was developed as an entirely novel method of measuring the potency of anti-inflammatory drugs; in practice it has proved to be a reliable screening method. It has not been possible to incorporate this paper into the general theme of the thesis.

Ganglionic blocking activity (pp. 270 - 277)

A series of quaternary ammonium sulphate compounds was investigated. The results confirmed that the maximum ganglionic blocking activity occurred with an optimum distance between the respective "onium" centres.

THE ANTITUBERCULAR PROPERTIES OF A SERIES OF THIOLS AND SULPHIDES

BY

P. ACRED and D. M. BROWN

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THE ANTITUBERCULAR PROPERTIES OF A SERIES OF THIOLS AND SULPHIDES

BY

P. ACRED AND D. M. BROWN

From the Beecham Research Laboratories Brockham Park, Betchworth, Surrey

(RECEIVED FEBRUARY 3, 1960)

The antitubercular activity of a series of thiols, dithiolans, thiol esters, dimercaptopropyl esters, and episulphides has been examined *in vitro* and *in vivo* in mice infected with the $H_{37}Rv$ strain of *Mycobacterium tuberculosis*. Most of the thiol compounds were inactive, although dimercaprol (2,3-dimercaptopropanol; B.A.L.) and a few closely related compounds showed slight activity *in vivo*, the only exception being 2,3-dimercaptopropyl chloride which was very active. The dithiolans were inactive, but some of the thiol esters were moderately active, in particular 2,3-di(acetylthio)propyl acetate and 1,2,3-tri(acetylthio)propane. The majority of the dimercaptopropyl esters had significant activity, the most active compounds being 2, 3 - dimercaptopropyl benzoate, 1, 3 - dimercapto - 2 - propyl benzoate, 2, 3 - dimercaptopropyl *o*-chlorobenzoate, and 2,3-dimercaptopropyl *p*-chlorobenzoate. All the *S*-acyl derivatives of 3-mercaptopropylene sulphide had good antitubercular activity, some being more active than streptomycin. The most active compound of the series was 3-(2-furoylthio)propylene sulphide. The activity of the compounds is believed to be due to their conversion *in vivo* to 3-mercaptopropylene sulphide and not due to the formation of ethanethiol. Slight deviation from the basic structure abolishes antitubercular activity.

Anderson and Chin (1947) first drew attention to the role of thiol compounds in experimental tuberculosis. These workers found that 2,3-dimercaptopropanol (dimercaprol; B.A.L.) 100 μg./ml. inhibited the growth of *Mycobacterium tuberculosis* grown in Dubos medium. Del Pianto (1950) reported the activity of 2-mercaptobenzothiazole in combination with salts of the *S*-esters of thiosulphonic acid. He suggested that the antitubercular activity of sodium ethyl thiosulphate was due to the formation of ethylmercaptan (ethanethiol). Solotorovsky and his colleagues [Brown, Matzuk, Becker, Conbere, Constatin, Solotorovsky, Winsten, Ironson and Quastel (1954); Solotorovsky, Winsten, Ironson, Brown and Becker (1954); and Solotorovsky, Winsten, Ironson and Brown (1956)] examined a series of ethylthio compounds, in particular *S*-ethyl L-cysteine, which was reported to be more active than *p*-aminosalicylic acid when administered in the diet. Other workers were unable to confirm these findings and further investigation revealed that the activity of *S*-ethyl-L-cysteine and other members of the series depended upon the formation of the volatile compound ethanethiol [Solotorovsky (1955) (1956); Solotorovsky, Ironson, Winsten (1956); Oginsky, Solotorovsky and Brown (1955) (1956)]. Kushner, Dalalian, Bach, Centola, Sanjurjo and Williams (1955) and Davies, Driver, Hoggarth, Martin, Paige, Rose and Wilson (1956) have investigated more recently the antitubercular activity of series of compounds related to ethanethiol. The latter authors considered that only compounds which were converted to ethanethiol were active.

We have investigated the antitubercular activity of dimercaprol and a number of related compounds and have found some active derivatives. The activity of the compounds does not appear to depend upon the formation of ethanethiol.

METHODS

The *organism* employed was the human strain of *Mycobacterium tuberculosis*, $H_{37}Rv$, reference National Collection of Type Cultures 7416.

Compounds to be tested for *in vitro* activity were treated according to their solubility. Aqueous solutions were used when possible, but compounds which were only sparingly soluble in water were dissolved in 1 ml. ethoxyethanol and diluted 1:20 with distilled water. This ether possesses some growth-inhibitory activity against *Mycobacterium tuberculosis* at a concentration of 1:20, but the amount of ethoxyethanol in the final test concentration was never allowed to exceed 1:200.

Serial concentrations of the dissolved compounds were made in Dubos medium. The tubes were inoculated with two drops of 1:10 concentration of a

suspension, standardized to Wellcome opacity tube 2, of a 10-day-old culture of *Mycobacterium tuberculosis* grown in Dubos, and incubated at 37°. Readings of growth were made at 7 days.

The activity is recorded as the lowest concentration of the compound (μg./ml.) inhibiting growth of the organism, that is, the minimal inhibitory concentration (M.I.C.).

In vivo *Tests.*—Male albino mice weighing 18 to 22 g. were infected by injecting intravenously 0.2 ml./20 g. of a suspension in saline of a 15-day-old culture of $H_{37}Rv$ strain of *Mycobacterium tuberculosis* grown in Proskauer and Beck medium. The suspension opacity was adjusted to a density equivalent to 4 mg. wet weight of organism/ml.

Compounds were administered daily in arachis oil (unless otherwise stated—see Tables) at doses of 1/2, 1/5, 1/10, and 1/20th of their approximate subcutaneous LD50 values to groups of mice, 10 to a group. The arachis oil solution acted as a depot from which the compounds were slowly absorbed. In each experiment one group received no treatment and acted

TABLE I
INACTIVE THIOLS

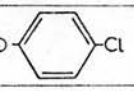
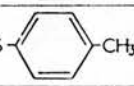
B.R.L. No.	Formula	B.R.L. No.	Formula
215	$CH_2(SH).CH_2.SH$	554	$[CH_2]_3(S.CH_2.CH(SH).CH_3)_2$
217	$CH_2(SH).CH_2.CH_2.SH$		
218	$CH_2(SH).CH_2.CH_2.CH_2.SH$	559	$CH_3.CH(SH).CH_2.S.[CH_2]_3.SH$
231	$CH_2(SH).CH(OH).CH_2.SH$	560	$CH_2(OH).CH_2.CH_2.S.CH_2.CH(SH).CH_3$
243	$CH_2(OH).CH_2.SH$	570	$CH_2(SH).CH_2(OH).CH_2.S.CH_2.CH(SH).CH_3$
244	$CH_2(SH).CH(SH).CH_3$	573	$CH_2(SH).CH_2.S.CH_2.CH_2SH$
248	$CH_2(SH).CH(SH).CH_2.SH$	576	$CH_2(SH).CH(OH).CH_2.CH_2SH$
259	$CH_2(OH).CH_2.CH_2.SH$	581	$CH_3.CH(SH).CH(SH).CH_2OH$
264	$CH_2(SH).CH(OH).CH_3$	584	$CH_2(SH).CH(SH).CH_2.S.CH_2.CH(SH).CH_3$
271	$CH_2(OH).CH(OH).CH_2.SH$	614	$CH_2(SH).CH(O.CO.CH_3).CH_2SH$
449	$CH_2(SH).CH(SH).CH_2.OC_2H_5$	622	$CH_2(SH).CH_2.CH(SH).CH_2O.CO.CH_3$
458	$CH_2(SH).CH(SH).CO.OCH_3$	633	$C(CH_2.SH)_2(OH)(CO_2H)$
476	$CH_2(SH).CH(SH).CH(OCH_3).CH_2.OCH_3$	639	$CH_2(OH).CH(SH).CH(SH).CH_2OH$
484	$CH_2(SH).CH(SH).CO_2H$	713	$CH_2(SH).CH(SH).CH_2.O.C_6H_5$
493	$CH_2(SH).CH(OH).CH(SH).CH_3$	735	
496	$CH_2(SH).C(CH_3)(OH).CH_2.SH$	756	
515	$CH_2(SH).CH_2.CH(SH).CH_2.OH$	765	$CH_2(SH).CH(OH).CH(SH).CH_2SH$
522	$CH_2(SH).CH(SH).CH(OH).CH_3$		
552	$CH_3.CH(SH).CH_2.S.CH_2.CH_2OH$		

TABLE II

ACTIVE THIOLS

All compounds were administered subcutaneously in arachis oil.

B.R.L. No.	Formula	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
—	Dimercaprol $CH_2(SH).CH(SH).CH_2OH$	50·0	100	9·5	7·0	—	—
479	$CH_2(SH).CH(SH).CH_2Cl$	6·25	100	—	24·0	16·0	9·0
489	$CH_2(SH).CH(SH).CH_2$ $\diagdown$ S $\diagup$ $CH_2(OH).CH(SH).CH_2$	25–50	>500	—	14·0	8·0	3·5
550	$CH_3.CH(SH).CH_2.S.CH_2.CH_2SH$	>100·0	200	10·0	7·0	2·5	—
555	$CH_2(OH).CH(SH).CH_2$ $\vert$ $CH_3.CH(SH).CH_2.S$	—	250	7·5	4·0	2·0	—
567	$CH_2(SH).CH_2(SH).CH(Cl).CH_3$	50·0	500	11·0	7·0	3·5	—
578	$CH_2(SH).C(CH_3)(Cl).CH_2SH$	>50·0	400	—	8·0	6·0	—
609	$CH_3.CH(SH).CH(SH).CH_2Cl$	>50·0	300	—	4·0	6·0	—
784	$CH_2(SH).[CH_2]_3.CH(SH).CH_2OH$	>50·0	500	—	4·0	0·5	—

as an infected control. Some compounds were tested for oral activity, either by administration by stomach tube or in the diet. The doses administered were again 1/2, 1/5, 1/10, and 1/20th of their approximate acute oral LD50. The water-soluble compounds were given in aqueous solution in a volume of 0.2 ml./20 g. and the insoluble solids as acacia suspension, 0.2 ml./20 g. When the compounds were given in the diet the solution or suspension was added to 250 g., so that the appropriate dose was contained in 5 g., that is, the average daily intake for one mouse. Daily dosing commenced in all cases 24 hr. after infection and continued until the twentieth day of the experiment. The TD50 values (median survival time) were determined by plotting the accumulative percentage mortality against time in days on log probit paper, and in cases where there was an increase in TD50 value over that of the control the results were analysed by the method of Litchfield (1949).

Toxicities.—Only approximate estimates of the acute LD50 values were obtained. The compounds were prepared as oil and water emulsions using acacia as the dispersing agent, and injected subcutaneously into groups of 5 mice per dose level.

RESULTS

Thiols

The inactive thiols are shown in Table I and the active thiols in Table II. The parent compound of the series, dimercaprol (2,3-dimercaptopropanol), and all the active thiols, apart from 2,3-dimercaptopropyl chloride (B.R.L. 479), were only slightly active *in vivo*. *In vitro* their activity was negligible. The compounds which were active possessed free thiol groups and a hydroxyl group, but not necessarily all compounds of this type were active. Acylation of the hydroxyl group or replacement of the hydroxyl group with a thiol, carboxyl or substituted carboxyl group abolished activity. Compounds having only one thiol group were inactive. If the hydroxyl group in the compounds having two vicinal thiol groups was replaced by a chlorine atom, activity was retained and might in fact be enhanced, for example, 2,3-dimercaptopropyl chloride (B.R.L. 479) was appreciably active *in vivo*, giving a considerable extension of the survival time. *In vitro* it had an

activity of 6.25 μg./ml., being the only member of this group which had a significant *in vitro* activity.

The chloro compounds B.R.L. 479, B.R.L. 567 and B.R.L. 609, which were prepared from the hydroxyl analogues and hydrochloric acid, may be contaminated with isomeric structures (Doyle, Holland, Mansford, Nayler, and Queen, 1960).

Dithiolans

Ring closure to form dithiolans resulted in complete loss of activity—Table III.

Thiol Esters

The inactive thiol esters are listed in Table IV and the active thiol esters in Table V. Some of the active compounds showed considerable *in vitro* activity. 2,3-Di(acetylthio)propyl acetate (B.R.L. 403) and 2,3-di(acetylthio)propanol (B.R.L. 464) are active at a concentration of 0.8 μg./ml. 1,2,3-Tri(acetylthiopropane (B.R.L. 411) was also very active *in vitro*. These three compounds also showed good activity *in vivo*. Increasing the size of the acyl group to a butyryl group, however, led to a considerable decrease both of *in vivo* and *in vitro* activity. The substitution of other radicals also led to a diminished activity.

TABLE III

DITHIOLANS

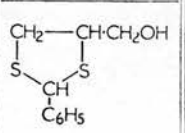
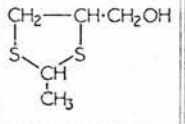
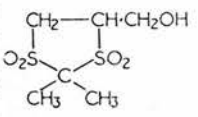
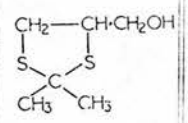
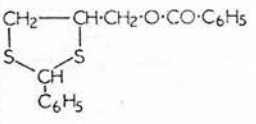
B.R.L. No.	Formula	B.R.L. No.	Formula
401		613	Glucose derivative of dimercaprol
412		647	
414		650	

TABLE IV

INACTIVE THIOL ESTERS

B.R.L. No.	Formula
450	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).CH_2.S.CO.CH_3$
471	$CH_2(O.CH_2COOH)CH(S.CO.CH_3).CH_2S.CO.CH_3$
543	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH_2.CH_2.S.CO.CH_3$
545	$CH_3CH(O.CO.CH_3).CH(S.CO.CH_3).CH_2.S.CO.CH_3$
568	$CH_2(S.CO.CH_3).C(CH_3)(O.CO.CH_3).CH_2.S.CO.CH_3$
583	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).CH_2.CH_2.S.CO.CH_3$
585	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH(S.CO.CH_3).CH_3$
640	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH(S.CO.CH_3).CH_2.O.CO.CH_3$
657	$C(CH_2.S.CO.CH_3)_2(O.CO.CH_3)(CO_2H)$
755	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).[CH_2]_4.S.CO.CH_3$
766	$CH_2(S.CO.CH_3).CH(S.CO.CH_3).CH(O.CO.CH_3).CH_2.S.CO.CH_3$
788	$CH_2(S.CO.CH_3).[CH_2]_8.CH(S.CO.CH_3).CH_2O.CO.CH_3$
826	$CH_2(S.CH_3).CH_2.S.CO.CH_3$

TABLE V

ACTIVE THIOL ESTERS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous suspension.

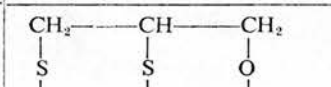
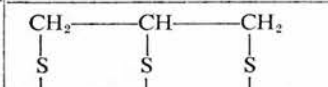
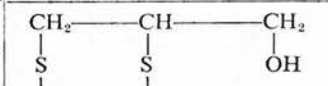
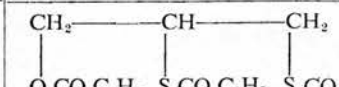
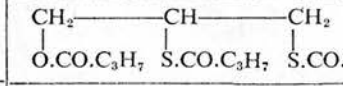
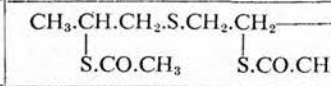
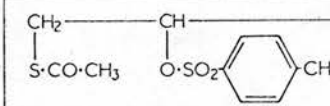
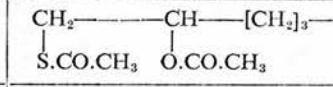
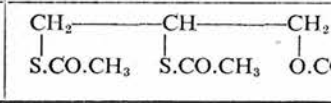
B.R.L. No.	Formula	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50		
				1/2	1/5	1/10
403		0·8	200	15·25	9·0	2·5
411		1·6	99	17·5	9·5	2·5
464		0·8	150	11·5	4·5	2·75
535		—	>500	15·0	2·0	2·0
536		6·25	500	2·0	−0·5	−0·5
562		6·25	500	2·5	−1·0	−1·5
655		6·25–12·5	>500	—	14·0	11·5
			150 (a)	—	2·0	3·0 (a)
758		>50·0	>500	—	4·0	1·5
877		—	>500	—	4·0	2·5

TABLE VI

INACTIVE DIMERCAPTOPROPYL ESTERS

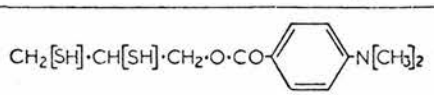
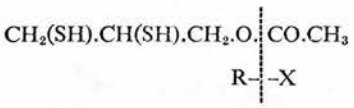
B.R.L. No.	Formula
646	$CH_2(SH).CH(SH).CH_2.O.CO.[CH_2]_{11}.CH_3$
694	

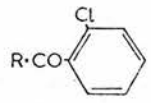
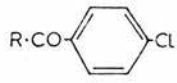
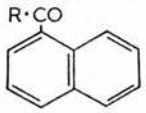
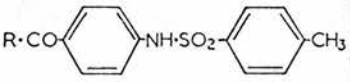
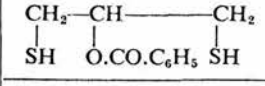
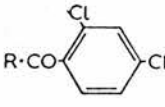
TABLE VII

ACTIVE DIMERCAPTOPROPYL ESTERS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous suspension, (*b*) orally in arachis oil.

General formula:



B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
463	R–CO.CH_3	3·125–6·25	78	15·0	8·0	4·4	—
504	R–CO.C_2H_5	12·5	150	13·0	4·0	—	—
510	R–CO.C_3H_7	25·0	400	13·0	8·5	1·5	—
587	R–CO.C_6H_5	12·5	>500	28·4	18·6	16·4	9·0
			600 (a)	5·2	2·5	1·25 (a)	—
			—	−3·5	11·5	0 (b)	—
636		25·0	>500	20·0	29·0	22·8	10·7
			500 (a)	6·5	2·0	2·5 (a)	—
637		12·5	500	54·0	29·0	16·0	11·25
638	R–CO.CH_2.C_6H_5	25·0	500	—	14·5	8·5	1·0
			>500 (a)	—	3·0	3·5 (a)	—
651		25·0	200	22·5	11·5	5·5	—
668		50·0	>500	—	9·5	2·5	—
670		>50·0	>500	—	8·0	0·5	—
671	R–CO.CH_2.S.C_2H_5	25·0	300		8·0	3·5	—
673	R–CO.CH_2.S.CH_2.C_6H_5	50·0	>500	14·0	10·5	5·0	--
			>500 (a)	3·0	1·0	−0·5 (a)	--
680		1·56	500	34·0	19·0	10·25	3·5
693	R–CO.$[CH_2]_2$.S.CH_3	25·0	500	—	8·5	6·0	—
707		25–50	150	—	3·0	0·75	--

Dimercaptopropyl Esters

The inactive compounds are given in Table VI and the active compounds in Table VII. When the hydroxyl group is esterified there is usually a considerable increase in antitubercular activity, particularly in compounds with vicinal thiol groups, and where the hydroxyl group is adjacent to these. 2,3-Dimercaptopropyl benzoate (B.R.L. 587), 2,3-dimercaptopropyl *o*-chlorobenzoate (B.R.L. 636) and 2,3-dimercaptopropyl *p*-chlorobenzoate (B.R.L. 637) are the most active of this particular type. The precise nature of the ester radical, however, does not seem to be of great importance in determining the degree of activity. All of the *O*-esters of dimercaprol were, however, comparatively inactive *in vitro*.

A notable exception to the general pattern is 2-mercapto-1-mercaptomethylethyl benzoate (B.R.L. 680), which has the two thiol groups separated by the ester group. This compound is active both *in vitro* and *in vivo*. The isomer 2,3-dimercaptopropyl benzoate (B.R.L. 587) is relatively inactive *in vitro*.

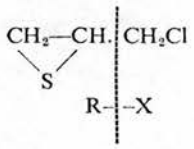
Episulphides

By eliminating the elements of water from thiol compounds containing a vicinal hydroxyl group

TABLE VIII

INACTIVE EPISULPHIDES

General formula:



B.R.L. No.	R.X
509	$R.CH_3$
511	$R.CH_2Cl$
512	$R.CH_2.O.C_2H_5$
513	$R.CH_2.S.C_2H_5$
514	$R.CH_2.N(C_2H_5)_2$
520	$R.CH_2.S.CO.NH.C_6H_5$
723	$R.CH_2.S.CH_3$

TABLE IX

ACTIVE EPISULPHIDES

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous solution, (b) orally in arachis oil, (c) in the diet.

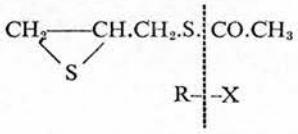
B.R.L. No.	Formula ($R=CH_2—CH—$, S)	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
482	$R.CH_2SH$	12·5	44 88 (a)	−1·5	−3·0	−3·0 (c)	—
				17·2	17·0	14·5	4·0
				3·0	1·25	1·0 (a)	—
				5·5	−1·0 (b)	—	—
528	$CH_3.CH——CH.CH_2SH$, S	100	200	—	2·5	1·5	—
553	$R.CH_2.S.S.CH_2.R$	—	500	3·5	18·0	14·0	—
577	$CH_2—CH.CH_2.CH_2SH$, S	50·0	400	—	4·5	6·75	—
599	$R.CH(SH).CH_3$	12·5–25·0	100	2·5	1·5	−1·0	—

TABLE X

EPISULPHIDES WITH *S*-ACYL GROUPS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (a) orally in aqueous suspension, (b) orally in arachis oil, (c) in the diet, (d) orally in acacia suspension, (e) subcutaneously in acacia suspension.

General formula:



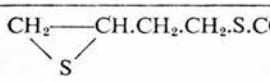
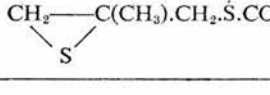
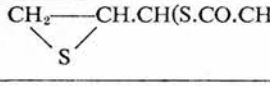
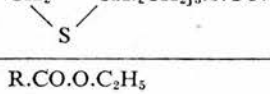
B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
459	$R.CO.CH_3$	0·4–0·8	45	17·25	14·0	12·0	4·5
			110 (a)	9·25	6·25	0·5 (a)	—
				3·5	2·0 (b)	—	—
				−2·5	−1·5	−2·5 (c)	—
540	$R.CO.C_2H_5$	1·56	100	21·0	17·5	11·0	—
546		—	300	14·5	5·25	0·5	—
561	$R.CO.C_3H_7$	6·25–12·5	100	14·5	13·5	7·0	—
571		50·0	300	2·5	2·7	1·5	—
580	$R.CO.CH_2.CH_2CO_2H$	25·0	100	— 7·5	11·5 3·5	5·0 4·0	—
			200 (a)	—	1·5	1·5 (a)	—
590	$R.CO.CH_2Cl$	25·0	60	16·0	17·5	16·3	6·0
			150 (a)	3·5	2·5	2·5 (a)	—
595		25–50	300	5·5	3·5	1·5	—
600	$R.CO.[CH_2]_2.CO.OCH_3$	1·56–3·125	100	17·5	6·5	12·0	—
601		3·125	>500	17·5	10·0	1·5	—
602	$R.CO.O.C_2H_5$	1·56	150	16·5	18·0	15·0	4·5
605	$R.CO.C_6H_5$	1·56	300	30·5	16·5	20·5	0
			400 (a)	4·0	2·5	2·0 (a)	—
615	$R.CO.CH_2.CO.O.C_2H_5$	3·125	75	22·5	11·0	8·0	—
617	$R.CO.CHCl_2$	25·0	150	—	17·5	10·0	5·0

TABLE X—*continued.*

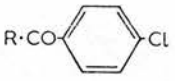
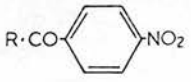
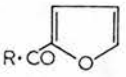
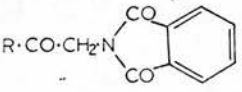
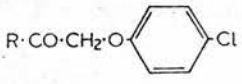
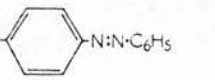
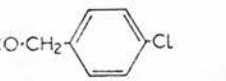
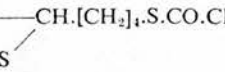
B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg/kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
625	$R.CO.CH_2.CH_2Cl$	6·25	300	—	19·5	17·5	—
629	$R.CO.CH_2.C_6H_5$	12·5	500	22·5	27·5	17·0	11·0
			>500 (a)	13·0	9·0	6·0 (a)	—
			—	—	4·0	0·5 (b)	—
635	$R.CO.CCl_3$	25·0	500	—	—	22·5	21·5
643	$R.CO.CO.O.C_2H_5$	12·5	100	17·5	13·5	10·5	—
649		50·0	200	29·5	12·5	2·5	—
			>1000 (d)	10·0	1·5	0·5 (d)	—
656		6·25	>500	29·5	15·0	11·0	—
			>500 (d)	16·25	5·2	2·75(d)	—
658		0·39	300	—	28·5	26·3	8·5
			500 (a)	—	7·0	0·5 (a)	—
664		>50·0	300	15·0	16·0	15·0	—
			1000 (d)	4·0	2·0	3·0 (d)	—
669		>50·0	200	—	5·5	2·5	—
672	$R.CO.CH(C_6H_5)_2$	>50·0	>500	6·0	1·0	1·0	—
677	$R.CO.CH_2.S.C_2H_5$	6·25	100		18·5	9·0	4·0
681	$R.CO.[CH_2]_{14}.CH_3$	>50·0	>500	4·0	0·5	−1·0	—
690	$R.CO.CH_2.NH.CO.O.CH_2.C_6H_5$	25·0	150	12·5	8·5	1·5	—
710	$R.CO.CH.[CH_2]_4.CH_2$	6·25–12·5	>500	—	34·5	23·5	1·5
711	$R.CO.[CH_2]_2.CO.CH_3$	25·0	100	—	23·5	—	—
			200 (a)	—	7·0	2·5 (a)	—
			—	—	2·5	2·0 (b)	—
721		3·125	>500		25·0	17·0	17·0 (e)

TABLE X—*continued.*

B.R.L. No.	R.X	*In vitro* Activity μg. ml.	LD50 mg kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1 2	1/5	1 10	1/20
727	$R.CO.CH_2.NH.CO.C_6H_5$	>50·0	400		17·0	16·0	1·5 (e)
728		3·125	>500	—	8·0	11·0	2·0 (e)
733		3·125–6·25	>500	—	21·0	13·0	5·5 (e)
746	$R.CO.O.CH_3$	6·25–12·5	50	—	8·5	4·5	—
747		25·0		—	12·5	9·0 (e)	—
748	$R.CO.CH(Cl).C_6H_5$	25·0	>500	—	2·5	0·5 (e)	—
753	$R.CO.O.C_4H_9$	3·125	>500	—	22·0	8·0	—
785		—	300	—	2·0	2·0	—

to form episulphides the compounds produced have enhanced antitubercular activity. The inactive compounds are shown in Table VIII and the active compounds in Tables IX and X. Since the synthetic method (Doyle, Holland, Mansford, Nayler, and Queen, *J. chem. Soc.*, in the press) was not structurally definite either or both of the preparations, B.R.L. 528 and B.R.L. 599, may be mixtures of the two isomeric structures. Of the compounds in Table IX only 3-mercaptopropylene sulphide (B.R.L. 482) and di(2,3-epithiopropyl) disulphide (B.R.L. 553) show good activity. On the other hand, the majority of episulphides with *S*-acyl groups (Table X) were highly effective chemotherapeutic agents. The compounds with an episulphide ring must, however, be comparatively simple in order to be active ; compounds with high activity being confined to *S*-acyl derivatives of 3-mercaptopropylene sulphide (B.R.L. 482). Substitution of methyl groups on any of the carbon atoms almost abolishes activity. The addition of groups other than simple acyl radicals also abolishes activity. The nature of the acyl radical, on the condition that it is a simple group, would not appear to be the main determining factor for antitubercular activity, but the acetyl (B.R.L. 459) and furoyl (B.R.L. 658) derivatives both have high *in vivo* and *in vitro* activities.

DISCUSSION

A large number of thiol compounds and related derivatives have been investigated for antitubercular activity, and many have proved to be active *in vivo*. There are, however, a number of discrepancies between the *in vitro* activity and the *in vivo* activity, and an explanation for this must be sought.

Since the majority of the episulphides are very active *in vivo*, it would appear that the episulphide structure is the necessary moiety for antitubercular activity. This view is supported by the investigations of Miles and Owen (1952) and Harding and Owen (1954), who have shown that acetylated thiols with a vicinal hydroxyl group in the presence of mild alkali are deacetylated with accompanying ring closure to form episulphides. The activity of the thiols and thiol *O*-esters can therefore be accounted for by such a mechanism. Further confirmation that an active derivative is formed has been obtained by the findings of Mansford and

Langley (to be published), who have shown that the addition of 3-acetylthiopropylene sulphide (B.R.L. 459) to serum leads to the formation of 3-mercaptopropylene sulphide (B.R.L. 482). If B.R.L. 482 is the active derivative, the absence of activity *in vitro* may be explained by the fact that it is an unstable compound and under the mild alkaline conditions of the *in vitro* test it forms inactive polymers.

The activity of the compounds tested is not due to their conversion to ethanethiol which has been shown to be the active agent produced by other thiol antitubercular agents (Oginsky, Solotorovsky, and Brown, 1955, 1956). We have been unable to detect ethanethiol in the expired air of animals injected with any of the compounds tested, nor has it been possible by using B.R.L. 587 containing radioactive ^{35}S to detect ^{35}S in the breath. Over 80% of the injected ^{35}S was recovered in the urine of rats in 24 hr. as sulphate.

The activity of the compounds which are active *in vitro* can possibly be explained by the assumption that the compound is absorbed on the surface of the organisms and the active metabolite (i.e., B.R.L. 482) is formed in close proximity to it and is therefore able to exert an antibacterial action before it is destroyed chemically. While there is little evidence for this theory, it does provide a likely explanation.

This explanation could also account for the apparent discrepancy between activities of 2,3-dimercaptopropyl benzoate (B.R.L. 587) and 1,3-dimercapto-2-propyl benzoate (B.R.L. 680) *in vitro* and *in vivo*. It is possible that B.R.L. 680 can be readily converted *in vitro* to the parent episulphide whereas B.R.L. 587 cannot. *In vivo*, however, the reaction may readily take place, with the result that both compounds are equally active. It is unlikely that the compounds are simply dealkylated to the hydroxyl compound without ring closure taking place, since the parent hydroxyl compound (B.R.L. 231) of B.R.L. 680 is highly toxic and completely inactive *in vivo*.

We wish to thank Mr. F. P. Doyle and his colleagues for preparing the compounds ; Dr. L. N. Owen for the supply of 3,4-dimethoxybutane-1:2-dithiol (B.R.L. 476), 1,4-dihydroxybutane-2:3-dithiol (B.R.L. 639) and 1,4-diacetoxy-2,3-(diacetylthio)butane (B.R.L. 640) ; Dr. J. H. C. Nayler for guidance on chemical nomenclature ; and Mr. D. Wright for technical assistance.

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THE ANTITUBERCULAR ACTIVITY OF 3-ACETYLTHIOPROPYLENE SULPHIDE AND 3-(2-FUROYLTHIO)PROPYLENE SULPHIDE

BY

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THE ANTITUBERCULAR ACTIVITY OF 3-ACETYLTHIO-PROPYLENE SULPHIDE AND 3-(2-FUROYLTHIO)PROPYLENE SULPHIDE

BY

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(RECEIVED FEBRUARY 3, 1960)

Two derivatives of 3-mercaptopropylene sulphide (B.R.L. 482), 3-acetylthiopropylene sulphide (B.R.L. 459) and 3-(2-furoylthio)propylene sulphide (B.R.L. 658), have been tested in mice for antitubercular activity. The compounds were administered daily in arachis oil by the subcutaneous route. When tested by a prolongation in survival time B.R.L. 459 was less active than streptomycin, while B.R.L. 658 was of the same order of activity. When assessed by the lesions present in the lungs after 14 days' treatment B.R.L. 658 was as active as isoniazid and B.R.L. 459 was of the same order of activity as streptomycin. Neither compound, however, cured an established infection and resistance developed rapidly to both compounds, both *in vitro* and *in vivo*. It is concluded that, in view of their adverse physical and pharmacological properties, the compounds are unsuitable for clinical use.

Acred and Brown (1960) have tested a series of new antitubercular compounds related to dimercaprol (2,3-dimercaptopropanol) and have produced evidence that the activity of the compounds depends on the formation of 3-mercaptopropylene sulphide. *S*-acyl derivatives of this compound possess considerable antitubercular activity. Two of the most active compounds, 3-acetylthiopropylene sulphide (B.R.L. 459) and 3-(2-furoylthio)propylene sulphide (B.R.L. 658), have been examined in greater detail.

METHODS

The methods and techniques for assessing antitubercular activity *in vitro* and *in vivo* were the same as those used by Acred and Brown in the previous paper. The organism employed in all tests was the human strain ($H_{37}Rv$) of *Mycobacterium tuberculosis*. In all tests the propylene sulphides were administered in arachis oil solution; streptomycin and isoniazid were given in aqueous solution.

Determination of the Extension of the Median Survival Time (TD50).—The antibacterial activity of the compounds was tested in mice (18 to 22 g.) infected with *Mycobacterium tuberculosis* and compared with that of streptomycin and isoniazid. Doses of one-fifth, one-tenth, one-twentieth and one-fortieth of the acute subcutaneous LD50 values were administered daily by the subcutaneous route for 20 days following the infection; the activity being expressed as the extension in days of the median survival time, over that of the infected control.

Lung Lesion Test.—As a further check on activity the procedure described by Solotorovsky, Winsten, Ironson, Brown, and Becker (1954) was employed. Ten mice (18 to 22 g.) were allocated to each group. The mice were infected intravenously with 0.8 mg. wet weight of *Mycobacterium tuberculosis*. The compounds were administered at one-fifth, one-tenth, one-twentieth and one-fortieth of the acute subcutaneous LD50 values. Treatment was stopped when 50% of the infected control animals died. On that day all surviving animals were killed and the lungs removed and kept in 10% formol saline for 48 hr. The degree of lung tuberculous involvement was scored in percentage for each group, the final result being expressed as the mean percentage tuberculous involvement.

Activity Against an Established Infection.—Groups of 10 mice were infected intravenously with 0.8 mg. wet weight *Mycobacterium tuberculosis*. Treatment was commenced after seven days, and continued until the twentieth day. Doses of the propylene sulphides equal to one-fifth and of streptomycin equal to one-tenth the acute subcutaneous LD50 were given by the subcutaneous route. The TD50 values were estimated and compared with an infected control group.

In vivo *Resistance.*—The propylene sulphides and streptomycin were administered daily by the subcutaneous route at one-twentieth of their acute subcutaneous LD50 values. Groups of 10 mice (18 to 22 g.) infected intravenously with 0.8 mg. wet weight *Mycobacterium tuberculosis* were used. When two or three mice remained in each group they were killed, and the lungs and spleens removed and homogenized. The tubercle bacilli were cultured from the

homogenates on Löwenstein-Jensen slopes and in Proskauer and Beck medium. For the next passage a further group of mice were infected with an inoculum prepared from the isolated organism grown on Proskauer and Beck medium. The *in vitro* sensitivity and virulence of the bacilli isolated at the first, third, and fifth passages were determined and compared with the results obtained for bacilli isolated at each passage taken from an infected, untreated control group of mice. The *in vitro* sensitivity was determined in Dubos medium. The virulence to mice was checked by infecting intravenously a group of 10 mice with 0.8 mg. wet weight of the organism isolated, and determining the TD50 in the absence of drug treatment.

Development of Resistance in vitro.—A suspension of a 10-day culture of *Mycobacterium tuberculosis*, standardized to Wellcome opacity tube 2, was diluted 1 : 10 with Dubos medium. Two drops of this suspension were added to tubes, each containing 2.5 ml. of serial dilutions of B.R.L. 459 and B.R.L. 658, streptomycin, and isoniazid in Dubos medium. Incubation was carried out at 37° for 7 days. The concentration of drug in μg./ml. which just inhibited growth (minimal inhibitory concentration) was noted. The inoculum for the subsequent passage was taken after a further 7 days' incubation from the tube containing the highest concentration of drug in which growth occurred. Passaging was continued until a high degree of resistance to the compounds had developed.

Results

Extension of Median Survival Time.—The results obtained are given in Table I. B.R.L. 459 is less active than streptomycin at all dose levels. B.R.L. 658 also appeared to be less active, but in 1 experiment with streptomycin a rather unusually long extension of the TD50 was recorded. If this test is not taken into account the activity of B.R.L. 658 and streptomycin would be of the same order. Isoniazid is considerably more active than both the compounds under test.

Lung Lesion Test.—The results obtained are given in Table II. When tested in this way B.R.L. 459 was of the same order of activity as streptomycin. At the dose levels employed all the lungs showed advanced tuberculous involvement. B.R.L. 658, on the other hand, was more active than streptomycin. At one-tenth of the subcutaneous LD50 dose the tuberculous involvement was slightly greater than minimal, while at one-twentieth of the LD50 there was only an average of 25% involvement. However, at one-fortieth of

Table I

THE ANTITUBERCULAR ACTIVITY OF 3-ACETYLTHIOPROPYLENE SULPHIDE (B.R.L. 459) AND 3-(2-FUROYLTHIO)PROPYLENE SULPHIDE (B.R.L. 658), STREPTOMYCIN AND ISONIAZID IN MICE

The extension of the TD50 value is the increase in survival time of the treated mice over the untreated mice, measured from the regression of percentage mortality against time on log-probit paper at the 50% mortality level. The *in vitro* activity was determined in Dubos medium seven days after inoculation.

Compound	Subcutaneous LD50 mg./kg.	*In vitro* Activity (M.I.C.) μg./ml.	Expt. No.	Extension in TD50 over Control—Days. Dose Fraction of Subcutaneous LD50			
				1/5	1/10	1/20	1/40
B.R.L. 459 ..	45·0	0·4–0·8	1		13·0	1·0	1·0
			2		7·0	2·5	2·5
			3		2·5	2·5	0
			4	14·0	12·0	4·5	0
B.R.L. 658 ..	300·0	0·8	1		17·0	9·0	3·0
			2		7·5	5·5	5·5
			3		4·5	0	1·0
			4	28·5	26·5	8·5	—
Streptomycin ..	520·0	0·4	1	—	16·5	9·0	4·0
			2	—	16·0	7·0	7·0
			3	59·0	38·0	15·0	6·5
			4	12·5	10·0	9·0	5·5
Isoniazid ..	182·0	0·05	1	—	89·0	44·0	38·0
			2			70·0	75·0

TABLE II

LUNG LESION TEST

The mice were killed and the lungs examined on the day 50% of the control mice died.

Compound	Expt. No.	% Lung Tuberculous Involvement. Dose Fraction of Subcutaneous LD50		
		1/10	1/20	1/40
B.R.L. 459	1	57	79	71
	2	100	100	100
	3	72	82	100
	Mean	76·3	87·0	90·3
B.R.L. 658	1	12	37	92
	2	8	27	50
	3	16	12	83
	Mean	12	25	75
Streptomycin	1		100	
	2		86	
	3		100	
	4		100	
	5	66	90	
	6	57	90	
	Mean	61·5	94·3	
Isoniazid	1	0		
	2	5	50	

the LD50 there was advanced involvement, but this was not a great deal worse than that seen in mice treated with one-tenth the subcutaneous LD50 of streptomycin. From the limited data with isoniazid, B.R.L. 658 appeared to have the same order of activity.

TABLE III

DELAYED THERAPY

Compound	TD50—Days. Dose Fraction of Subcutaneous LD50	
	1/5	1/10
B.R.L. 459	21·0	
B.R.L. 658	20·5	
Streptomycin ..		100% survival after 30 days
Infected control (no treatment)	20·0	

Action Against Established Infection.—Neither B.R.L. 658 nor B.R.L. 459 gave any protection to mice which had been infected 7 days previously. On the other hand, streptomycin had a pronounced antitubercular action ; all the mice still survived 30 days after the infection (see Table III).

In vivo *Resistance.*—The *in vitro* sensitivities of the bacilli isolated from mice at the end of the first, third and fifth passages are given in Table IV.

TABLE IV

VIRULENCE AND *IN VITRO* SENSITIVITIES OF *MYCOBACTERIUM TUBERCULOSIS* PASSAGED IN MICE

Mice infected with *Mycobacterium tuberculosis* were treated daily with one-twentieth the subcutaneous LD50 of B.R.L. 459, B.R.L. 658, and streptomycin for twenty days. At the end of each passage the organism was isolated on Löwenstein and Jensen slopes from the mice and the *in vitro* sensitivity (minimum inhibitory concentration—μg./ml.) determined in Dubos medium. The virulence was determined by infecting groups of ten mice and estimating the TD50 in the usual manner.

Passage	1		3		5	
Compound	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days
B.R.L. 459	0·78	19·0	1·56–3·13	18·0	12·5	12·0
Control ..	1·56	18·0	—	13·0	0·78	15·0
B.R.L. 658	0·78	22·0	6·25	20·0	3·13	14·0
Control ..	0·39	18·0	—	13·0	0·39	15·0
Streptomycin	0·78	—	0·39	14·0	0·2	19·0
Control ..	0·78	18·0	—	13·0	0·2	15·0

Resistance of the bacilli to B.R.L. 459 and B.R.L. 658 had developed markedly by the end of the fifth passage, while no change in the sensitivity to streptomycin was detectable. In every instance the virulence of all the isolated bacilli was fully maintained.

In vitro *Resistance.*—Resistance induced by *in vitro* transfer to B.R.L. 658 and B.R.L. 459 developed with exceptional rapidity (see Fig. 1). The minimal inhibitory concentration at the commencement of the test for B.R.L. 658 and B.R.L. 459 was 0.39 μg./ml., and within five passages this

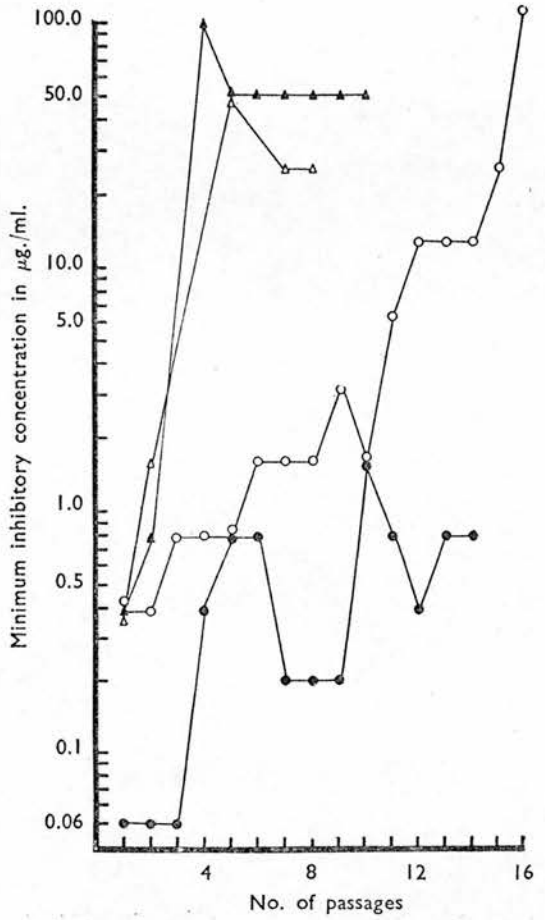

FIG. 1.—Development of resistance of *Mycobacterium tuberculosis* ($H_{37}Rv$) in Dubos medium to B.R.L. 459, B.R.L. 658, streptomycin, and isoniazid. ▲ B.R.L. 459. △ B.R.L. 658. O Streptomycin. ● Isoniazid.

rose to 50 to 100 μg./ml. for B.R.L. 459, and 25 to 50 μg./ml. for B.R.L. 658. In the control series resistance to streptomycin began to develop within twelve passages, the minimal inhibitory concentration rising from 0.39 to 6.25 μg./ml. With isoniazid resistance began to develop at the fourth passage, but did not increase to the degree shown by the propylene sulphides.

DISCUSSION

B.R.L. 459 and B.R.L. 658 are highly active antitubercular compounds when tested in mice infected with the $H_{37}Rv$ strain of *Mycobacterium tuberculosis*. B.R.L. 658 is the more active of the two compounds, comparing favourably with streptomycin by the TD50 test, and is of the same order of activity as isoniazid by the lung lesion test. B.R.L. 459 shows a corresponding difference in relative activity to streptomycin and isoniazid by the two methods. The difference in relative activity observed using the two methods is probably due to the compounds having a bacteriostatic and not bactericidal action *in vivo*. In the TD50 test administration of the compounds is stopped after 20 days when a generalized lethal infection develops. On the other hand, in the lung lesion test the mice are still under treatment when they are killed. The growth of the organism is well suppressed at this stage and the lungs are practically devoid of lesions. The lung lesion test therefore gives a more favourable impression of relative activity.

Resistance to the compounds develops exceptionally rapidly *in vitro* and also *in vivo*. They are also incapable of curing an established infection and are ineffective orally. When administered daily over a period of six months to guinea-pigs infected with *Mycobacterium tuberculosis* they give rise to ulcers of such severity that they render an assessment of antitubercular activity in guinea-pigs impracticable. In addition, the compounds are odorous, oily, water-insoluble liquids which are unstable in the presence of water and in mild alkaline conditions. In spite, therefore, of their initial highly promising activity in mice, their adverse physical and pharmacological properties would seem to rule out their possible clinical use.

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CHEMOTHERAPEUTIC STUDIES ON A NEW ANTIBIOTIC —BRL.1241

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THE discovery and isolation of 6-aminopenicillanic acid (Batchelor et al. 1959) has made it possible to synthesise a wide range of penicillins with modified properties. One of these, BRL.1241,* has proved highly effective against penicillin-resistant staphylococci.

Methods and Results

In the following experiments BRL.1241 and penicillin G were administered as their sodium salts.

General Pharmacology

In mice and cats no toxic symptoms were observed with single doses of 2.5 g. per kg. intravenously, and in mice and rats with doses of 4 g. per kg. subcutaneously. When BRL.1241 was administered daily to rats for twelve weeks at doses of 100 and 500 mg. per kg. subcutaneously, and to dogs for four weeks at 250 mg. per kg. twice daily subcutaneously, no biochemical, hæmatological, or histological abnormalities were noted. Apart from the discomfort of the injection, the compound was well tolerated. 1% and 10% solutions were injected intramuscularly and intradermally into rats, guineapigs, and rabbits without any local irritation. Similarly, a 1% solution applied daily to the eyes of rabbits was without effect. Administration to anæsthetised cats at a dose of 500 mg. per kg. had no effect on blood-pressure and respiration.

Blood-levels in rabbits and dogs after intramuscular injection resembled those following injection of penicillin G. The mechanism of excretion in chicks was also similar to that of penicillin G. Like penicillin G, BRL.1241 penetrates the blood-brain barrier only with difficulty. It is protein-bound to a lesser degree than penicillin G.

* 'Celbenin' is the registered trade name of Beecham Research Laboratories Ltd. for BRL.1241.

C.D.$_{50}$ VALUES FOR BRL.1241 AND PENICILLIN G DETERMINED IN MICE (10 PER DOSE LEVEL)

Organism	C.D.$_{50}$ (mg. per kg.)	
	BRL.1241	Penicillin G
Klebsiella pneumoniæ	Inactive	Inactive
Salmonella typhimurium	Inactive	Inactive
Diplococcus pneumoniæ	10·0	1·0
Staphylococcus pyogenes Smith	1·3	0·2
Staph. pyogenes 52–75 (penicillin-resistant) ..	100·0	Inactive

Protection Tests

The protective effect of BRL.1241 was determined in mice infected with *Klebsiella pneumoniæ*, *Salmonella typhimurium*, *Diplococcus pneumoniæ*, *Staphylococcus pyogenes* Smith and *Staph. pyogenes* 52-75, the last organism being highly resistant to penicillin G (minimal inhibitory concentration 125 μg. per ml.).

Of the first four organisms, serial dilutions of 18-24-hour cultures were prepared in 5% hog gastric mucin to enhance the virulence, and 0·5 ml. volumes were injected intraperitoneally. Deaths were recorded over four days and the number of organisms killing 50% of the mice was estimated (L.D.$_{50}$). The mice under test were injected with a hundred times this number of organisms.

Penicillin G and BRL.1241 were administered subcutaneously to groups of 10 mice in graded doses to the log base 4, in 0·2 ml. saline immediately following the infection. The mice were observed for four days and the deaths were recorded daily. The percentage deaths were plotted against log dose, and the dose of compound (mg. per kg.) giving protection to 50% of the mice (C.D.$_{50}$) was read off from the graph. The estimate of the C.D.$_{50}$ against *Staph. pyogenes* 52–75 was carried out in exactly the same way, except that 0·5 ml. of a 1/2 dilution in 5% hog gastric mucin of an overnight culture was used to infect the animals.

The results obtained are given in the table.

A further estimate of the in-vivo effect against penicillin-resistant organisms was carried out using the method described by Selbie and O'Grady (1954). Groups of 10 mice (18–22 g.) were infected by injecting into the muscle sheath of the thigh 0.2 ml. of a 1/2 dilution of an overnight growth of *Staph. pyogenes* Russell (type 80). BRL.1241 in doses of 50, 100, and 200 mg. per kg. and penicillin G 200 mg. per kg. were administered subcutaneously daily for three days, the first injection being given immediately following the infection. An infected control group

received no treatment. The maximum thigh diameter of each mouse was measured with callipers on the first, third, and eighth day after the infection. The results (fig. 1) clearly demonstrate a graded response to dose. The activity against other resistant staphylococci has been confirmed.

The activity of BRL.1241 was further confirmed by a subsequent series of experiments, using the technique of Selbie and O'Grady (1954), in which groups of 10 mice were infected with two strains of staphylococci. *Staph. pyogenes* Russell (resistant to penicillin G) was injected into the muscle sheath of the right hind leg, and *Staph. pyogenes* 2187 (sensitive to penicillin G) was injected into the left hind leg. Because untreated mice usually died from this double infection, two control groups were used, one receiving the sensitive strain in the left leg, and the other receiving the resistant strain in the right leg. The thigh diameters were measured as described above. Penicillin G and BRL.1241 were administered subcutaneously at a dose of 100 mg. per kg. daily for three days.

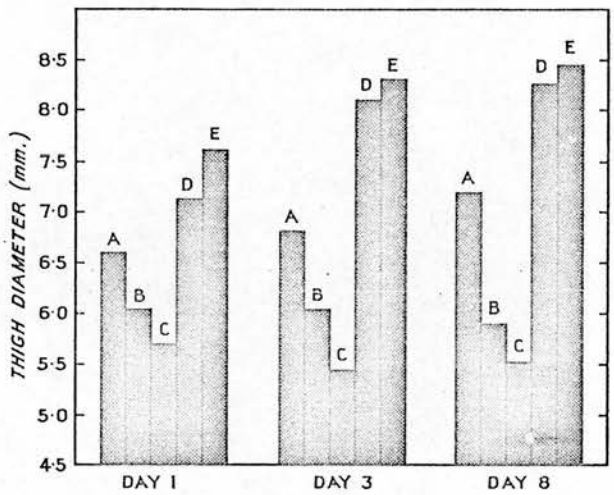

Fig. 1—Mean thigh diameters of mice (10 per group) infected intramuscularly with *Staph. pyogenes* Russell (penicillin-resistant). The following doses were administered subcutaneously daily for three days:

Group A:—BRL.1241. 50 mg. per kg.
„ B:— „ 100 „ „ „
„ C:— „ 200 „ „ „
„ D:—Penicillin G. 200 „ „ „
„ E:—Untreated infected control.

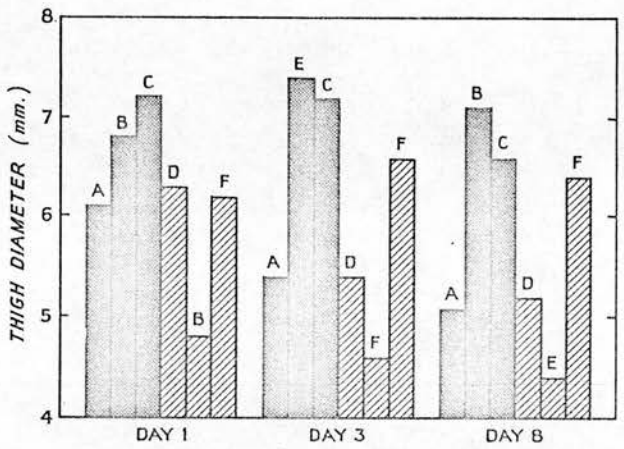

Fig. 2—Mean thigh diameters of mice (10 per group) infected intramuscularly with *Staph. pyogenes* Russell (penicillin-resistant) in the right hind limb and *Staph. pyogenes* 2187 (penicillin-sensitive) in the left hind limb.

BRL.1241 and penicillin G, 100 mg. per kg., were administered subcutaneously daily for three days.

Blocks A, B, and C are the mean thigh diameters of the limb infected with the resistant staphylococcus, and D, E, and F are the mean diameters of the limb infected with the sensitive staphylococcus. Group 1 (A and D) received BRL.1241, group 2 (B and E) received penicillin G, and group 3 (C and F) was the untreated infected control.

The results (fig. 2) show the effectiveness of BRL.1241 in mice infected simultaneously with both resistant and sensitive staphylococci. Penicillin G is completely inactive against the resistant strain, but more effective than BRL.1241 against the sensitive strain.

Conclusion

BRL.1241 is remarkably free from toxic actions. It can be given in high doses intramuscularly or intravenously. The blood-levels obtained after an intramuscular injection in rabbits and dogs follow a pattern very similar to that obtained after penicillin G. Like penicillin G, it requires large doses before it will penetrate into the cerebrospinal fluid.

BRL.1241 does not combine with serum protein to the same degree as penicillin G. This, however, does not seem to have influenced the cross-allergenic response in patients already sensitive to penicillin (Stewart et al. 1960).

It is highly effective against staphylococci resistant to penicillin G, and the results obtained clearly demonstrate

the activity in vivo. Though BRL.1241 is not as effective as penicillin G against sensitive strains of staphylococci, its activity is still considerable. In mice infected with both penicillin-sensitive and penicillin-resistant strains of staphylococci, it was equally active against the two organisms, whereas penicillin G was active only against the sensitive strain. This is in agreement with the in-vitro findings of Knox (1960), Rolinson et al. (1960), Stewart et al. (1960) and Thompson et al. (1960).

We wish to thank Prof. C. A. Keele for his general interest and helpful discussions; Dr. A. A. G. Lewis, physician to the Connaught Hospital, Walthamstow, under whose skilled guidance the dog studies were conducted; and Mr. F. P. Doyle and his colleagues for the preparation of the compound. In addition, we wish to acknowledge the excellent technical assistance of Mr. D. Wright and Mr. M. J. Wilson.

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PHARMACOLOGY OF METHICILLIN

BY

P. ACRED, D. M. BROWN, D. H. TURNER and D. WRIGHT

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PHARMACOLOGY OF METHICILLIN

BY

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(*Received March* 7, 1961)

The pharmacology of a new antibiotic methicillin, 6(2:6-dimethoxybenzamido)-penicillanic acid, which is effective against staphylococci resistant to penicillin G, has been investigated. It is free from acute and chronic toxic effects, except that some pain may be caused following intramuscular injection. It is poorly absorbed orally, but after intramuscular injection the concentrations in the serum and in tissues are very similar to those found with penicillin G. It is excreted by the kidneys both by renal tubular secretion and glomerular filtration. It is also excreted in the bile in very high concentrations, the ratio of concentration in the bile to the blood being approximately 2.5 times that of penicillin G. From a study of the metabolism of the drug it is calculated that 75% is eliminated unchanged in the urine and that the remainder is probably destroyed after the excretion into the intestine via the bile.

With the isolation of 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959), it is now possible to synthesize a wide range of new penicillins having modified antibacterial properties. One of these new derivatives, 6-(2:6-dimethoxybenzamido)penicillanic acid, has been shown to possess remarkable stability to staphylococcal penicillinase, and recent clinical reports have confirmed its effectiveness in the treatment of infections caused by staphylococci resistant to penicillin G (Douthwaite & Trafford, 1960; Knox, 1960; Stewart, Harrison & Holt, 1960; and Stewart, Nixon, Coles, Kesson, Lawson, Thomas, Mishra, Mitchell, Semmens & Wade, 1960). Preliminary pharmacological and chemotherapeutic findings have been reported by Brown & Acred (1960). Full details of the pharmacological evaluation are now presented.

METHODS

In all experiments penicillin G and methicillin were administered as their pure sodium salts. The antibiotics were assayed by the cup-plate technique using *Sarcina lutea* as the test organism. The zone diameters obtained for the control dilutions of the antibiotics were plotted against the log of the concentration, and from the regression line obtained the concentrations of the antibiotics in the specimens were estimated by interpolation. The appropriate dilutions of the controls and samples were made in phosphate buffer *p*H 7.0 (M/20) except in the experiments where serum concentrations were determined, in which case the controls were prepared in serum.

Acute toxicities

The acute toxicity was determined in male albino mice (18 to 22 g) after intravenous, subcutaneous and oral administration, and subcutaneously in rats (150 to 200 g). The effect on respiration and the electrocardiogram was studied in guinea-pigs and cats during the slow intravenous infusion of the antibiotic.

Prolonged administration

The effects of prolonged administration were investigated in rats and dogs.

(i) *Rats.* Two groups of 12 male rats, each animal weighing 80 to 100 g, were injected subcutaneously 5 days per week with methicillin at doses of 100 and 500 mg/kg in a volume of 0.1 ml./100 g body weight for a period of 12 weeks. A group receiving 0.1 ml./100 g body weight normal saline subcutaneously acted as controls. Daily food intake and the weight of each rat was recorded. Weekly records of the red and white blood cell counts and qualitative tests for sugar and protein in the urine were performed. Haemoglobin determinations and spectroscopic examinations of the blood were made on the first, sixth and twelfth week of the test.

At the end of 6 weeks, 6 rats from each group were killed and the remaining rats killed at 12 weeks. The weights of the livers, spleens, kidneys, testes and adrenals were recorded, and specimens of liver, spleen, kidney, lung, thyroid, heart, duodenum, stomach, pancreas, adrenal, testis and bone marrow were removed for histological examination.

(ii) *Dogs.* Methicillin (250 mg/kg) was administered subcutaneously twice daily for a period of 4 weeks to two dogs. The following biochemical and haematological estimations were made at weekly intervals: haemoglobin (g%), packed cell volume, total white cell count, blood urea, serum alkaline phosphatase, zinc sulphate turbidity and serum globulin and albumin. A differential blood cell count was carried out at the end of the first and final week of the test.

Local irritant action

Ten per cent. and 1% solutions were injected intramuscularly and intradermally into rats and guinea-pigs (3 per group). The solutions were administered in a volume of 0.1 ml. intramuscularly into the hind legs, and 0.05 ml. intradermally on a shaved area on the backs. After 24 hr the area of the injection was examined and the skin and subcutaneous tissues removed for histological examination.

The effect of methicillin on the eye was examined in a group of 3 rabbits. A 1% solution in normal saline was dropped into a pocket formed by pulling out the lower left eyelid. The solution was held over the eye for 1 min. Saline was similarly applied to the right eye. The eyes were examined at 1, 2, 4, 8 and 24 hr afterwards for signs of irritation.

Blood pressure and respiratory effects

The carotid blood pressure of 5 cats anaesthetized with a 4% urethane/1% chloralose mixture (5 ml./kg intravenously) was recorded manometrically on a smoked drum. Respiration was recorded by a lever connected by means of a thread which was sewn to the skin over the xiphisternum. Methicillin in physiological saline was administered intravenously through the femoral vein at intervals of 5 min.

Absorption

(a) *Oral*

(i) *Rabbits.* 100 and 500 mg/kg doses of methicillin were administered orally to groups of 5 rabbits. Blood samples for assay were removed from the lateral ear vein at 1, 2, 4 and 6 hr.

(ii) *Absorption from small intestine of rats.* The bile ducts of rats, 5 to a group, were cannulated as described by Harrison *et al.* (1960). The rats were restrained in close-fitting wire cages and the total bile and urine collected at 2, 4, 6 and 24 hr after the administration of 100 mg/kg methicillin directly into the duodenum. Blood specimens were taken from the tail vein at 1, 3, 5 and 24 hr after injection.

(b) *Intramuscular—serum and urine concentrations in dogs*

Methicillin and penicillin G were injected intramuscularly at a dose of 5 mg/kg, and blood and urine specimens were taken at intervals up to 6 hr after administration.

The blood specimens were removed by means of a sterile syringe from the radial vein, allowed to clot at room temperature and the serum transferred to sterile tubes and frozen. The urine was removed from the bladder, by means of a polythene cannula, at the same time as the blood samples were taken.

Distribution and elimination

(a) *Serum and cerebrospinal fluid concentrations in rabbits*

(i) *Serum concentrations.* Two groups of 5 rabbits were given intramuscular injections, in the hind leg, of 100 mg/kg methicillin and 100 mg/kg penicillin G ; 0.5 ml. samples of blood were removed from the lateral ear veins at 1, 2, 4 and 6 hr after administration. The samples were allowed to clot at room temperature and the serum was removed and kept at 4° C until assayed (at the end of 6 hr).

(ii) *Cerebrospinal fluid concentrations.* The cerebrospinal fluid concentrations were determined in anaesthetized rabbits after administration of 100 and 500 mg/kg methicillin intramuscularly. The rabbits were anaesthetized with urethane (1 g/kg intraperitoneally). The cerebrospinal fluid samples were withdrawn from the cisternum magnum by means of a sterile syringe and needle. Blood samples were taken from the lateral ear vein at the time of taking the cerebrospinal fluid samples. The concentrations of methicillin in the serum and the cerebrospinal fluid were assayed as before.

(b) *Blood levels and bile excretion in the anaesthetized rat*

Two groups of 5 rats (230 to 265 g) were anaesthetized with 1.0 ml./kg pentobarbitone intraperitoneally. After laparotomy the bile duct was cannulated with polythene tubing (0.4 mm internal diameter). 100 mg/kg methicillin and penicillin G was administered intramuscularly to each of the groups. The bile was collected over 30 min periods in sterile tubes which were cooled in iced water. 0.1 ml. samples of arterial blood were obtained at hourly intervals up to 5 hr from the right carotid artery and diluted with 0.4 ml. normal saline containing 100 units heparin.

(c) *Hen*

The technique described by Sperber (1949) was employed. The cloacal mucosae of 6-month-old laying Sussex hens were anaesthetized with a few drops of 2% cinchocaine hydrochloride in normal saline. A polythene funnel was held in position over each ureteral opening by stitching the mucosa to the free edge of a rubber washer which surrounded the head of the funnel. Short polythene tubes were inserted into the stems of the funnels and the urine from each kidney collected in sterile tubes which were cooled in iced water.

The antibiotics were dissolved in 2.0 ml. of 0.9% saline and injected into the muscles of the left leg. Probenecid, which blocks renal tubular secretion, was administered intravenously at a dose of 100 mg. The probenecid solution was prepared from 0.5 g tablets (Merck Sharpe & Dohme). Five tablets were powdered and triturated with 10 ml. 0.5 N sodium hydroxide and the *p*H adjusted to 7.4 with 1.0 N hydrochloric acid (2 to 3 ml.). The volume was made up to 100 ml. with 0.9% saline, giving a solution containing 25 mg probenecid per ml. Four ml. of this solution was injected into a wing vein 10 min prior to the administration of the antibiotic.

In order to increase urine flow, 100 ml. of warm tap water was given by crop tube at the commencement of the experiment. Samples of urine were taken for assay at 10 min intervals for the first hour after dosing and thereafter at hourly intervals up to 6 hr.

In the experiments 4 hens were employed, each of which received the following treatments on four separate occasions: (i) 100 mg methicillin ; (ii) 100 mg penicillin G ; (iii) 100 mg methicillin + 100 mg probenecid ; (iv) 100 mg penicillin G + 100 mg probenecid.

(d) *Distribution in tissues*

100 mg/kg methicillin was administered intramuscularly to groups of 10 rats, 1 group being killed at 0, ½, 1, 2, 4, 12 and 24 hr after the injection. The rats were exsanguinated by cutting the throat and the blood collected. The urine and faeces from each group were

collected and the amounts recorded. The following organs and tissues were removed and weighed: liver, spleen, kidney, lung, small intestine, large intestine and muscle at site of injection. The carcass was then weighed. All specimens were homogenized in a Waring blender. Appropriate dilutions of the homogenate were made with phosphate buffer *p*H 7.0 and specimens of the homogenate assayed. The total amount of antibiotic in each specimen was calculated. The specimens which were known to be contaminated were not specially treated, as we found that in general the assay figures obtained were not unduly influenced.

Serum binding—dialysis

Five ml. of bovine, horse or human serum containing 5 mg of either methicillin or penicillin G was placed in cellophane bags (Visking tubing ¾ in.) and suspended in 20 ml. sterile saline at 10° C for 48 hr. At the end of the period of dialysis the amounts of the antibiotic outside and inside the tubing were assayed. Five tubes were prepared of each antibiotic in each experiment.

RESULTS

Toxicities

(i) *Acute*

Methicillin when given intravenously to mice at doses up to 2.5 g/kg produces no signs of toxicity. When larger doses are given, 3 to 5 g/kg, occasional mild clonic convulsions are produced within 5 min of administration. These give way to a phase which may persist up to 4 hr in which the mice exhibit a loss of muscle tone. If disturbed during this phase, a mild muscular spasm is induced causing extension of the limbs. In the range 4 to 5 g/kg some deaths may occur due to cardiac and respiratory failure. With some animals respiration may cease for periods up to 10 sec, but this is followed by complete recovery. No toxic symptoms were noted in mice and rats given 4 g/kg of the antibiotic orally or subcutaneously.

(ii) *Prolonged administration*

(a) *Rats.* No untoward toxic symptoms were noted in the rats treated with methicillin. Post-mortem examination did not reveal any abnormalities and the

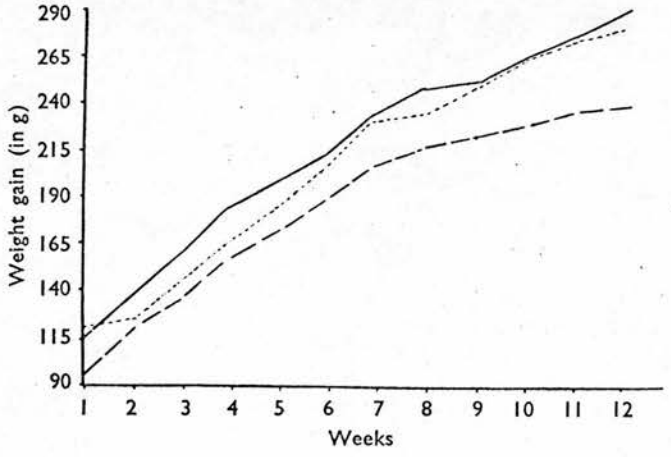

Fig. 1. Mean growth curves of rats—12 to a group—following daily intramuscular administration of (*a*) 500 mg/kg methicillin (——); (*b*) 100 mg/kg methicillin (- - - -); (*c*) 1.0 ml./kg normal saline (— — —).

histology of the organs examined was normal. The growth curves are shown in Fig. 1. The animals which received the antibiotic gained more weight than the controls.

(b) *Dogs.* No abnormal signs were observed in any of the tests carried out. The histology of the essential organs was normal.

Local irritant action

Macroscopically there was no evidence of tissue damage, except following the 10% intradermal injection, when there was a slight induration and erythema of the skin lasting 24 to 48 hr. Histologically there is a marked inflammatory reaction in the rat skin with necrosis of epidermis and hair follicles, following intradermal injection of 10% methicillin. A comparable inflammatory reaction is seen in the muscle. The changes in both skin and muscle of rats are much less marked with the 1% solution. The changes in the guinea-pig skin and muscle are similar; as severe in the skin at 10% but less marked in the muscle at that strength. The skin and muscle of guinea-pigs injected with the 1% solution showed no appreciable abnormality.

Cardiovascular and respiratory effects

When administered by slow intravenous injection to 5 guinea-pigs, 5 g/kg methicillin in saline given over a period of 25 min had no observable effect on the respiration or the electrocardiogram. When similarly administered to cats, 2.5 g/kg given over a 10 min period had no effect on the heart or respiration. In individual doses ranging from 10 to 500 mg/kg, methicillin was again without effect. The blood pressure response of cats to intravenous doses of adrenaline, noradrenaline, acetylcholine and histamine was unaffected by doses of 500 mg/kg methicillin. The pressor response to carotid occlusion was also unaffected by methicillin.

Absorption

(a) *Oral*

(i) *Rabbits.* No blood levels were detectable after 100 mg/kg, but significant blood levels were attained following administration of 500 mg/kg, viz., 3.0, 1.6, 1.6 and 1.45 μg/ml. at 1, 2, 4 and 6 hr respectively after administration.

(ii) *Absorption from small intestine of rats.* The concentration of methicillin found in the bile, urine and the blood after the intraduodenal administration of 100 mg/kg to rats is given in Table 1. Practically no blood levels were detectable, and only a total of 6.5% of the antibiotic was recovered—1.8% from the bile, 3.7% from the urine and 0.97% from the intestine.

(b) *Intramuscular—serum and urine concentrations in dogs*

The mean serum concentrations of penicillin G and methicillin obtained in three dogs after intramuscular injection of 5 mg/kg are shown in Fig. 2. Methicillin gives a slightly lower maximum blood concentration (2.9 μg/ml.) than penicillin G (4.05 μg/ml.). The peak also occurs slightly more in advance of penicillin G, but the fall-off in serum concentration of both antibiotics occurs at much the same rate. The excretion in the urine of both antibiotics is also very similar, 29% of penicillin G and 33% of methicillin being recovered in the urine in 6 hr.

TABLE 1

ABSORPTION OF METHICILLIN IN THE CONSCIOUS RAT AFTER INTRADUODENAL DOSING WITH 100 MG/KG

Rat	Dose in mg	Bile: % of dose excreted (hr) 0–2	2–4	4–6	6–24	Urine: % of dose excreted (hr) 0–2	2–4	4–6	6–24	Blood levels: concentration μg/ml. (hr) 1	3	5	24	% of dose remaining in intestine after 24 hr	Total % recovered
B	30	0·12	0·12	0·17	0·23	1·30	0·60	0·16	2·45	0·28	0·39	0	0	3·40	8·55
D	34	1·02	0·31	0·42	0·86	2·06	0·69	0·29	1·87	0	0	0	0	0·74	8·26
E	27	0·57	0·29	0·27	0·12	1·04	0·79	0·41	0·51	0	0	0	0	0	4·00
F	26	0·12	0·63	0·42	0·48	0·39	0·95	0·85	1·88	0	0	0	0	0·71	6·43
G	28	0·64	0·75	0·56	1·14	0·93	0·24	0·42	0·70	0	0	0	0	0	5·38
Mean % of dose excreted		0·49	0·42	0·36	0·56	1·14	0·66	0·42	1·48					0·97	6·52
Cumulative % excreted		0·49	0·91	1·27	1·83	1·14	1·80	2·22	3·70						

TABLE 2

DISTRIBUTION OF METHICILLIN IN THE RAT

Seven groups of 10 rats were injected intramuscularly with 100 mg/kg methicillin. One group was killed at the end of each time period. The mean concentration of methicillin is expressed in μg/g wet weight of tissue (column a) and the concentration ratio between the tissues μg/g wet weight to serum μg/ml. is shown in column b. The urinary excretion is expressed as a % of the dose administered

	0 hr		0·5 hr		1 hr		2 hr		4 hr		12 hr		24 hr	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Liver			32·8	1·01	23·8	0·79	12·9	2·36	4·1	22·8	0	—	0	—
Spleen			4·5	0·14	2·03	0·07	1·47	0·27	0	0	0·68	—	0	—
Kidneys			122·1	3·76	88·8	2·94	24·1	4·41	2·91	16·17	0·22	—	0	—
Lungs			15·6	0·42	8·8	0·29	2·34	0·43	1·36	7·56	0·19	—	0	—
Small intestine			74·9	2·3	133·9	4·43	149·6	27·4	54·7	304·0	1·05	—	0	—
Large intestine			5·31	0·16	6·84	0·23	7·76	1·42	31·41	14·45	14·5	—	0	—
Injection site	1,620·0		821·0	25·27	332·5	11·01	74·65	13·67	7·8	43·34	0·64	—	0	—
Carcass	2·0		11·8	0·29	9·39	0·31	2·52	0·46	3·7	20·6	0·71	—	0	—
Serum			32·5	1·0	30·2	1·0	5·46	1·0	0·18	1·0	0	—	0	—
Urine % excreted			13·39		32·6		42·8		48·9		74·6		68·4	

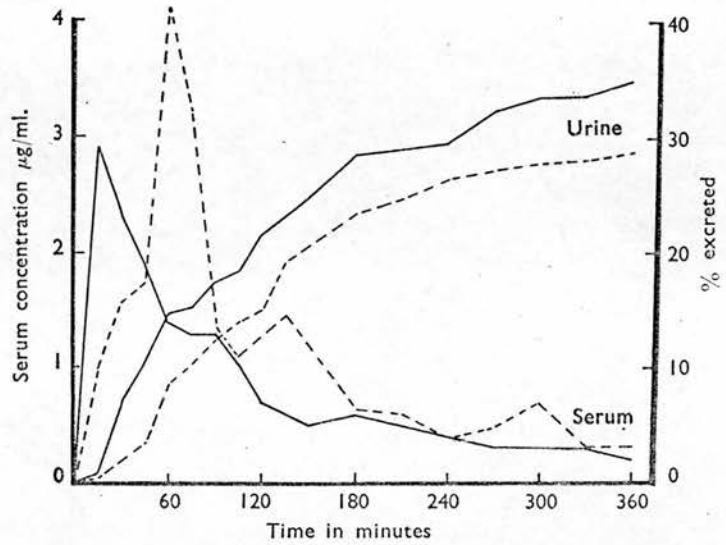

Fig. 2. Serum concentration and urinary excretion in dogs following intramuscular administration of 5 mg/kg methicillin (——) and penicillin G (– – –).

Distribution and elimination

(a) *Serum and cerebrospinal fluid concentrations in rabbits*

(i) *Serum concentrations.* The serum concentrations of penicillin G and methicillin after intramuscular injection to each of 5 rabbits are given in Table 3. The serum concentration picture of each antibiotic is similar.

TABLE 3

SERUM CONCENTRATIONS IN RABBITS RECEIVING 100 MG/KG METHICILLIN AND PENICILLIN G INTRAMUSCULARLY

	Serum concentration μg/ml. at hr after dosing			
Compound	1	2	4	6
Methicillin	12·5	3·65	0·51	0
	18·0	3·65	0·3	0
	14·0	4·2	0·74	0
	18·0	5·0	0·34	0
	14·0	6·2	1·3	0
Mean	15·3	4·48	0·64	0
Penicillin G	11·0	5·1	2·65	1·13
	9·0	5·0	1·17	0·5
	16·0	10·5	1·0	0·1
	10·5	4·7	0·48	0
	9·8	6·1	1·15	0·23
Mean	11·26	6·28	1·29	0·39

(ii) *Cerebrospinal fluid concentration.* The concentration of methicillin obtained in the serum and cerebrospinal fluid after the intramuscular administration of 100 and 500 mg/kg is shown in Table 4. The concentration of antibiotic in the cerebrospinal fluid is approximately 100 times less that the concentration appearing in the

TABLE 4

MEAN CONCENTRATIONS IN SERUM AND CEREBROSPINAL FLUID OF RABBITS (3 PER DOSE LEVEL) AFTER INTRAMUSCULAR ADMINISTRATION OF METHICILLIN

Dose mg/kg	Tissue	Concentration μg/ml. at hr after dosing				
		0·5	1	2	3	6
100	C.s.f.	0·4	0·35	—	—	0
	Serum	34·0	24·0	—	—	0
500	C.s.f.	1·17	2·3	3·6	4·4	0
	Serum	130	136	288	130	1·0

serum. The time of maximum concentration of methicillin in the serum does not coincide, however, with the time of maximum concentration in the cerebrospinal fluid, the latter occurring somewhat later.

(b) *Blood levels and bile excretion in the anaesthetized rat*

The blood picture of the anaesthetized rat after the administration of penicillin G and methicillin is shown in Fig. 3. The blood levels of both antibiotics are similar and both are markedly concentrated in the bile, though at the end of 6 hours both the blood and bile levels of methicillin were a little higher than those of penicillin G.

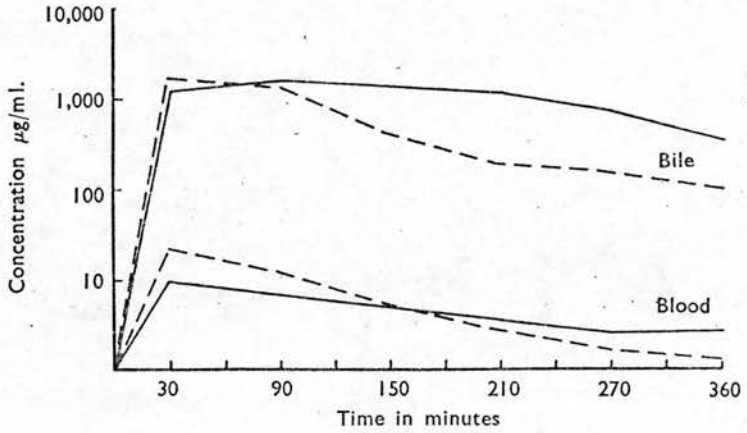

Fig. 3. Mean concentration in blood and bile of methicillin (——) and penicillin G (– – –) after intramuscular administration of 100 mg/kg of each antibiotic to groups of 5 anaesthetized rats.

(c) *Hen*

The excretion in urine of penicillin G and methicillin from the right and left kidneys following intramuscular administration into the left limb of 100 mg/kg of each antibiotic is illustrated in Fig. 4. The concentration of both antibiotics in the urine from the left kidney is considerably greater than the concentration in the urine from the right kidney. However, after the administration of 100 mg probenecid intravenously the concentration of both antibiotics in the urine from the right and left kidneys becomes identical.

(d) *Distribution in tissues*

The tissue concentrations of methicillin expressed as μg/g of wet weight of tissue are given in Table 2 (column a). In order to show the degree of concentration in the

various tissues and organs, the results have also been expressed as a ratio of the serum concentration (column b). Thirty min after the dose was given, the concentration occurring in the kidneys was approximately four times, that in the small intestine about double, and that in the liver about the same as that found in the serum. Lower concentrations occurred in the lungs, spleen and large intestine. The concentration of antibiotic in the serum falls off rapidly, but the concentration level in the other tissues apart from the spleen falls off more slowly, with the result that at the end of 4 hr the concentrations in the tissues are higher than those found in the serum. At the end of 12 hr there are only trace amounts left in most of the tissues,

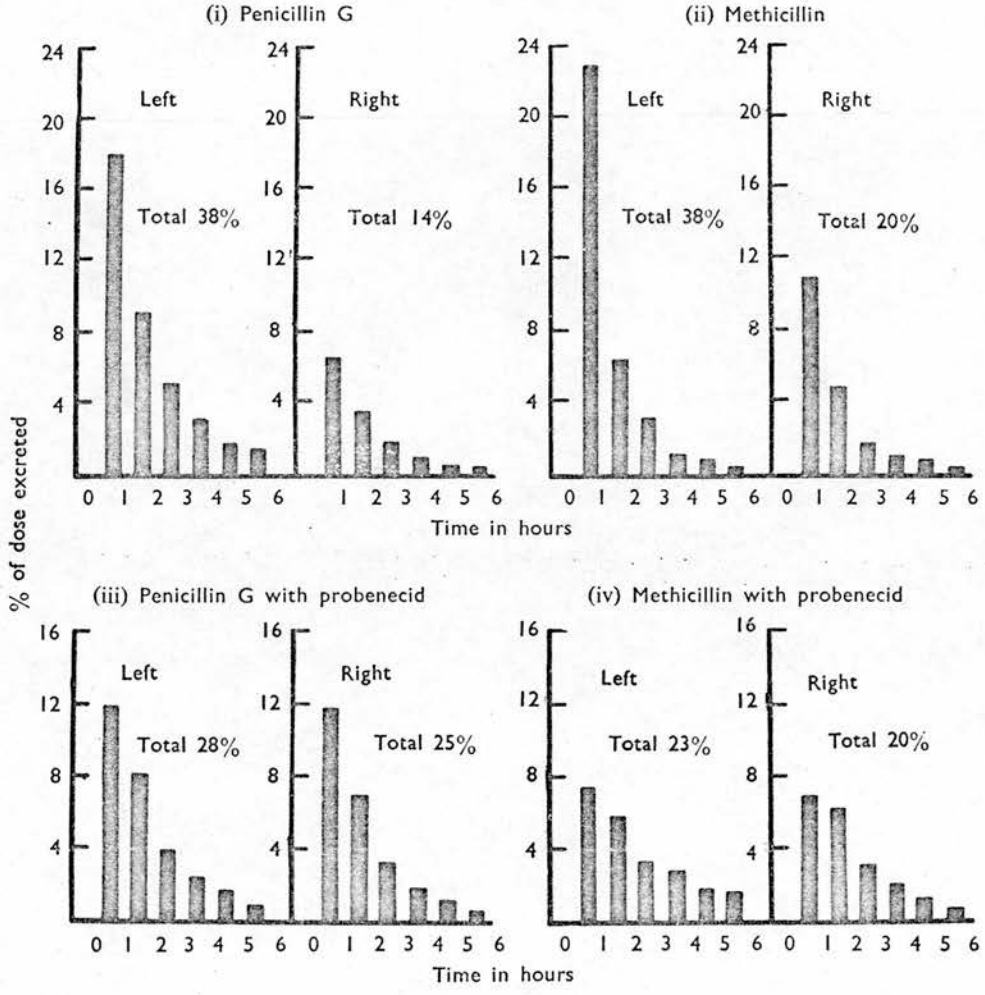

Fig. 4. Excretion of methicillin and penicillin G in the hen. The mean urinary excretion from the left and right kidneys of 4 hens each of which received, on four separate occasions: (i) 100 mg penicillin G; (ii) 100 mg methicillin; (iii) 100 mg penicillin G+100 mg probenecid; (iv) 100 mg methicillin+100 mg probenecid. The antibiotics were given intramuscularly and the probenecid intravenously.

none being detectable in the serum and liver. After 24 hr no antibiotic was detectable in any of the tissues. The urinary excretion is expressed as a percentage of the dose administered, a total of 74.6% being recovered in the first 12 hr. A total of 73.7%, 73.5%, 66.6%, 55.3%, 54.3%, 75.1% and 68.4% of methicillin was recovered from all tissues and body fluids at 0, 0.5, 1, 2, 4, 12 and 24 hr after administration.

Serum binding

Methicillin is considerably less bound to horse, bovine and human serum than penicillin G, the figures being respectively for each of the sera 10.2%, 4.5% and 17.7% for methicillin, and 21.0%, 27.3% and 43.4% for penicillin G.

DISCUSSION

An antibiotic should preferably be free from toxic effects. In this respect methicillin has remarkably few unwanted reactions. No toxic effects were noted in animals after large single intravenous doses. In one experiment a dose of 18 g was administered by slow intravenous infusion to a guinea-pig before serious toxic effects became apparent. When administered over a prolonged period both rats and dogs tolerated large doses, the only observable reaction being some discomfort at the time of injection. This is in agreement with clinical reports (Douthwaite & Trafford, 1960 ; Knudsen & Rolinson, 1960).

Methicillin is poorly absorbed from the gastro-intestinal tract, but it is well absorbed following parenteral administration, only small quantities remaining at the site of injection 12 hr afterwards. Good blood levels are obtained, and the antibiotic is distributed throughout the tissues of the body. There is a high concentration of antibiotic in the kidney, while the concentration in the liver is of the same order as that found in the serum for the first hour, but higher concentrations than those in the serum are subsequently found. The high concentrations appearing in the small intestine are probably accounted for by the presence of bile.

The total quantity of methicillin recovered from the intact animal falls steadily over the first 4 hr to 55%, but fully 75% is eventually recovered in the urine after 12 hr. It would seem that the antibiotic is taken up by some of the tissues in a non-assayable form and is then slowly released and then excreted in the urine.

That methicillin does combine with protein is shown by the dialysis experiments. There is considerable binding with serum protein, but it is less than that found with penicillin G. The degree of binding varies according to species, and it is possible that some of the antibiotic is more firmly bound to one type of protein than another and therefore it is more difficult to assay in this state. The antibiotic may be slowly released from this combination. Otherwise, it is difficult to postulate why there is a " loss " which is subsequently recovered in the urine.

The blood level picture and urinary excretion found for methicillin and penicillin G are practically identical, the pattern being similar in rabbits, dogs and conscious rats. In the anaesthetized rat the excretion is delayed, 50 to 55% of the antibiotics appearing in the urine of conscious rats during a 4 hr period, while only 25 to 35% is recovered in 6 hr from an anaesthetized animal, and as a consequence the blood

level is more prolonged. In view of this difference it would seem preferable to use non-anaesthetized animals for metabolism studies.

A marked difference is seen between the bile concentrations of methicillin and penicillin G. Comparison of the bile/blood ratio for the two antibiotics obtained in rats shows that methicillin is concentrated in the bile fully 2 to 2.5 times more than penicillin G. A total of 15 to 20% of the injected dose of methicillin is excreted in the bile—this we confirmed in both the anaesthetized and conscious rat—while 15% of penicillin G is similarly excreted. In view of the greater concentration ratio of methicillin from the blood to the bile, it might be expected that the difference between the amounts excreted would be more, but the initial blood levels of penicillin G are higher and thus proportionately a larger quantity appears in the bile.

In the normal animal one would expect that a considerable proportion of the antibiotic would be reabsorbed from the intestine after being excreted in the bile. However, the experiments where 100 mg/kg was injected into the duodenum of rats indicate that absorption even in the small intestine is poor—only 3.7% is recovered from the urine and 1.8% from the bile in 24 hr. The bulk of the dose appears to be destroyed, as only 0.97% can be accounted for in the intestine after 24 hr. From the observations on the distribution of the antibiotic in the intact animal one can therefore account for practically the whole of the dose administered, 75% being excreted in the urine and 15% lost in the intestine. We have found no evidence that 6-aminopenicillanic acid is formed in the body from methicillin; neither have we found that methicillin is excreted in a conjugated form in the bile as reported by Harrison *et al.* (1960).

The mode of elimination by the kidneys is well demonstrated in the hen experiment. Birds possess a renal portal system, and the venous return from the hind limbs is shunted through the parenchyma of the renal tubules. Therefore, when a substance is injected into a hind limb and both renal tubular secretion and glomerular filtration take place, the concentration of the substance excreted is greater in the urine from the ipsilateral kidney than in the urine from the contralateral kidney. If only glomerular filtration takes place, then the concentration in the urine excreted from both kidneys is identical. In our experiments the ipsilateral kidneys excreted more of the antibiotics than the contralateral kidneys, and blockage of renal tubular secretion resulted in equal concentrations of the antibiotics appearing in the urine from both kidneys. Therefore methicillin and penicillin G are excreted by the same mechanism, both glomerular filtration and renal tubular secretion taking place.

We wish to thank Dr A. C. Thackray, of the Bland Sutton Institute of Pathology, for expert histological comment; Dr A. A. G. Lewis, physician to the Connaught Hospital, Walthamstow, under whose skilled guidance the dog studies were conducted; and Mr F. P. Doyle and his colleagues for the preparation of the compound.

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"PENBRITIN"—A NEW BROAD-SPECTRUM ANTIBIOTIC

PRELIMINARY PHARMACOLOGY AND CHEMOTHERAPY

BY

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AND

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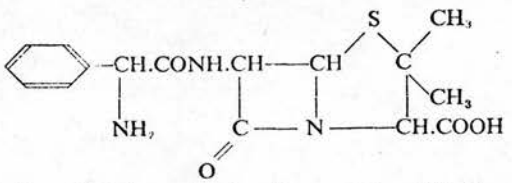
None of the present family of synthetic or natural penicillins possess appreciable activity against Gram-negative bacilli. Those penicillins which are absorbed orally are inactive in this respect. Since the isolation of 6-aminopenicillanic acid (Batchelor *et al.*, 1959), one of the principal objectives has been the development of a new penicillin which is absorbed orally and is effective against Gram-negative bacilli. Doyle and his colleagues have now synthesized a new penicillin, "penbritin" (6[D(−) - α - aminophenylacetamido]penicillanic acid), which, from the biological studies carried out, has been found to possess these properties.



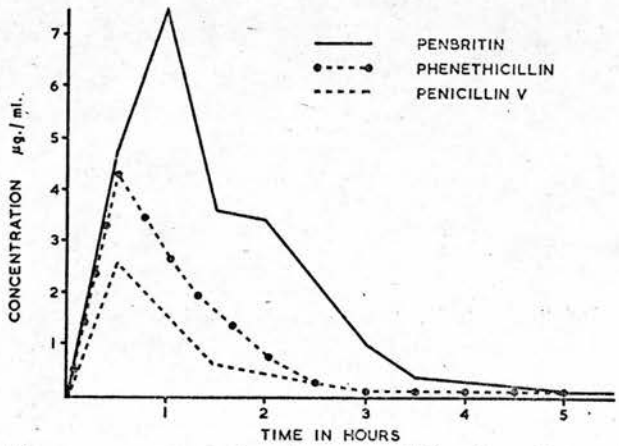
Penbritin; 6[D(−)-α-aminophenylacetamido]penicillanic acid.

Pharmacology

Penbritin (B.R.L. 1341) is non-toxic to mice, rats, dogs, and cats. Single doses up to 5 g./kg. have been administered orally and subcutaneously to mice and rats without observable toxic effects. Intravenously, the

amount given has been limited by solubility, but as much as 2 g./kg. has been administered to mice without lethal effects, although muscle tremor, slow respiration, and mild clonic convulsions have been noted. Similarly, in cats no adverse effects on the blood-pressure and respiration were seen with doses up to 100 mg./kg. intravenously. A total of 3 g./kg. was administered in divided doses over a period of one hour without toxic effects.

When administered orally to groups of 12 rats over a period of 12 weeks at doses of 500 and 100 mg./kg. no effect on the growth was observed; neither were any biochemical, haematological, or histological abnormalities seen. Penbritin, when administered orally to three dogs, 250 mg./kg. twice daily over a period of four weeks, likewise produced no toxic symptoms, apart from slight loosening of the stools. It gives good blood-levels after oral administration. In dogs, maximum blood-levels of 7.2 μg./ml. have been obtained after 20 mg./kg., the peak occurring one hour after dosing. On the other hand, after 20 mg. phenethicillin and penicillin V per kg. the peak levels which occurred in 30 minutes were 4 and 2.6 μg./ml. respectively (see Chart). In addition, the blood-levels were more persistent with penbritin, this effect having also been noted in humans (Knudsen *et al.*, 1961). After absorption the antibiotic is evenly distributed throughout the body tissues apart from kidney and liver, where higher concentrations than



Mean serum concentrations in groups of five dogs after oral administration of 20 mg. penbritin, phenethicillin, and penicillin V per kg.

in the serum are found. The antibiotic which is excreted in urine and bile is concentrated considerably in these fluids. The concentration in the bile is 300 times and in the urine 800 times that found in the blood. Penbritin is eliminated by the kidney by renal tubular secretion and glomerular filtration in a similar manner to penicillin G and methicillin (Acred *et al.*, 1961).

Chemotherapy

Penbritin has been tested in mice infected with *Staphylococcus aureus* (Smith), *Streptococcus pyogenes* (group A), *Salmonella typhi-murium*, and *Klebsiella pneumoniae*, and compared with penicillin V, phenethicillin, tetracycline, and chloramphenicol. The activity was assessed by determining the dose which cured 50% of a group of 10 mice infected with one of the above organisms. Penbritin, penicillin V, and phenethicillin all had the same order of activity against *Staph. aureus* by the oral route, the CD50 values in each case being 0.3 mg./kg., but tetracycline was fully 10 times less effective, having a CD50 of 5.2 mg./kg.; chloramphenicol was ineffective orally and subcutaneously. Penbritin, penicillin V, and phenethicillin were highly active against *Str. pyogenes*, giving an oral CD50 of 0.1 mg./kg.; tetracycline gave a CD50 of 0.5 mg/kg., while in this instance chloramphenicol was active with a CD50 of 3.2 mg./kg.

Against the Gram-negative infections penbritin was surprisingly effective when compared with tetracycline and chloramphenicol. The CD50 values against *Salm. typhi-murium* were, respectively, 19, 125, and 310 mg./kg., and against *Kleb. pneumoniae* 12, >400, and 165 mg./kg. Penicillin V and phenethicillin were completely inactive.

Conclusions

Penbritin is an effective non-toxic oral penicillin possessing a wide spectrum of activity. Animal studies indicate that it is better absorbed and gives more prolonged blood levels than penicillin V and phenethicillin. Its effectiveness against infections due to staphylococci and streptococci is equal to that of the existing oral penipenicillins. In this respect there is agreement with the *in vitro* activities (Rolinson and Stevens, 1961). On the other hand, the activity of penbritin *in vivo* against infections produced by Gram-negative organisms is considered to

be greater than that found with tetracycline and chloramphenicol. This is in marked contrast to the *in vitro* titres, where only small differences are found.

We thank Mr. F. P. Doyle and his colleagues for the preparation of penbritin.

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PHARMACOLOGY AND CHEMOTHERAPY OF AMPICILLIN—A NEW BROAD-SPECTRUM PENICILLIN

BY

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PHARMACOLOGY AND CHEMOTHERAPY OF AMPICILLIN—A NEW BROAD-SPECTRUM PENICILLIN

BY

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The pharmacology and chemotherapy of a new penicillin, 6[D(−)-α-aminophenylacetamido] penicillanic acid, are described. It is non-toxic, is absorbed orally and is distributed throughout the body in a manner similar to other penicillins. It is eliminated unchanged from the body in high concentrations in the bile and urine. Almost all of the antibiotic can be accounted for in the urine and intestinal contents 2 hr after intramuscular administration but not after oral administration. It is concluded that the antibiotic is not metabolized within the body. Studies with infected animals show that it is as effective as the existing oral penicillins against *Staphylococcus pyogenes* Smith (penicillin sensitive), *Streptococcus pyogenes* Group A and *Diplococcus pneumoniae*. It is ineffective against penicillin-resistant *Staphylococci*. When tested in mice infected with the gram-negative organisms, *Salmonella typhimurium* and *Klebsiella pneumoniae*, it was considerably more active than tetracycline and chloramphenicol.

There is a need for a penicillin which is effective orally and having activity against both gram-negative and gram-positive organisms. Benzylpenicillin, although lacking in toxicity, is unfortunately not absorbed after oral administration and its activity against gram-negative organisms is limited. Doyle, Nayler & Smith (1961) have synthesized a new penicillin, ampicillin (6[D(−)-α-aminophenylacetamido] penicillanic acid), which largely overcomes these disadvantages. The preliminary pharmacology and chemotherapy has been reported by Brown & Acred (1961). Full details of the pharmacological and chemotherapeutic evaluation are now presented.

METHODS

In all experiments benzylpenicillin was administered as its sodium salt. Ampicillin, which was 84% pure, was also administered as its sodium salt, all doses being given in terms of the pure compound. The antibiotics were assayed by the cup-plate technique using *Sarcina lutea* as the test organism. The zone diameters obtained for the control dilutions of the antibiotics were plotted against the log of the concentration, and from the regression line obtained the concentrations of the antibiotics in the specimens were estimated by interpolation. The appropriate dilutions of the controls and samples were made in phosphate buffer *p*H 7.0 (M/20) except in the experiments where serum concentrations were determined, in which case the controls were prepared in serum.

Chromatographic studies were carried out using 1 cm wide strips of Whatman no. 1 filter papers, 53 cm long with the origin at 11 cm. The solvent used was ethyl acetate-isopropanol-water in the proportions 4:2:1. 100 ml. of solvent was used in the trough of each tank.

All tanks were lined with Whatman no. 3 mm filter paper. The chromatograms were run from 16 to 20 hr at 20° C after equilibrating for at least 1 hr.

Detection of the penicillin on the chromatograms was carried out by placing the strips on agar plates seeded with *Sarcina lutea* and incubating overnight at 30° C. The location of the penicillin was seen as clear zones of growth inhibition on the plates. Control strips were set up using an aqueous ampicillin solution.

Acute toxicities

The acute toxicity was determined in male albino mice (Edwards strain—18 to 22 g) after intravenous, subcutaneous and oral administration, and in Sprague-Dawley rats (150 to 200 g) after subcutaneous and oral administration.

Prolonged administration

The effects of prolonged administration were investigated in rats and dogs.

(i) *Rats.* Doses of 500 and 100 mg/kg were administered orally by stomach tube to groups of 12 male rats, 5 days per week for a period of 12 weeks. The dose was administered as a freshly prepared aqueous solution in a volume equivalent to 0.5 ml./100 g body weight. The control group received an equivalent volume of tap water. Daily food intake and the weight of each rat was recorded. Weekly records of the red and white blood cell counts and qualitative tests for sugar and protein in the urine were performed. Haemoglobin determinations and spectroscopic examination of the blood were made on the first, sixth and twelfth week of the test. At the end of 12 weeks all the rats were killed and the weights of the livers, spleens, kidneys, testes and adrenals were recorded. Specimens of liver, spleen, kidney, lung, thyroid, heart, duodenum, stomach, pancreas, adrenal, testis and bone marrow were removed from 6 animals in each group for histological examination.

(ii) *Dogs.* Ampicillin (250 mg/kg) was administered orally twice daily for a period of 4 weeks to 2 dogs. The following biochemical and haematological estimations were made at weekly intervals: haemoglobin (%), packed cell volume, total white cell count, blood urea, serum alkaline phosphatase, zinc sulphate turbidity and serum globulin and albumin. A differential blood cell count was carried out at the end of the first and final week of the test.

Local irritant action

5% and 1% solutions were injected intramuscularly and intradermally into guinea-pigs (3 per group). The solutions were administered in a volume of 0.1 ml. intramuscularly into the hind legs, and 0.05 ml. intradermally in a shaved area on the back. After 24 hr the area of the injection was examined and the skin and subcutaneous tissues were removed for histological examination.

The effect of ampicillin on the eye was examined in a group of 3 rabbits. A 25% solution in normal saline was dropped into a pocket formed by pulling out the lower left eyelid. The solution was held over the eye for 1 min. Saline was similarly applied to the right eye. The eyes were examined at 1, 2, 4, 8 and 24 hr afterwards for signs of irritation.

Blood pressure and respiratory effects

The carotid blood pressures of 5 cats anaesthetized with a 4% urethane/1% chloralose mixture (5 ml./kg intravenously) were recorded manometrically on a smoked drum. Respiration was recorded by a lever connected to a thread which was sewn to the skin over the xiphisternum. Ampicillin in physiological saline was administered intravenously through the femoral vein at intervals of 5 min.

Absorption, distribution and elimination

(a) Absorption—oral and intramuscular

(i) *Rabbits.* 100 mg/kg doses of ampicillin and phenoxymethyl penicillin were administered orally and intramuscularly to groups of 5 rabbits. Blood samples for assay were removed from the lateral ear vein at 1, 2, 4 and 6 hr.

(ii) *Dogs.* Ampicillin was administered orally and intramuscularly to groups of 5 dogs. A dose of 5 mg/kg was given intramuscularly and a dose of 20 mg/kg orally. Blood specimens were taken up to 5 hr after administration. Following the intramuscular administration, a comparison was made with benzylpenicillin, and following the oral administration a comparison was made with phenoxymethyl penicillin. The blood specimens were removed by means of a sterile syringe from the radial vein and were allowed to clot at room temperature and the serum was transferred to sterile tubes and frozen.

(iii) *Rats—intestinal administration.* Male rats (290 to 430 g) were starved overnight. After anaesthetizing with ether, laparotomy was performed and the bile duct was cannulated with polythene tubing (0.40 mm bore). The latter was passed through a small aperture in the abdominal wall and the skin and muscle incisions were sutured. The animals were then dosed and placed in close-fitting restraining cages of wire mesh. Blood, bile and urine specimens were obtained at intervals throughout the 24 hr following injection of ampicillin direct into the duodenum (100 mg/kg, 1.0 ml./100 g body weight). At the termination of the experiment, homogenized samples of the following were assayed: (*a*) small intestine from pylorus to ileo-caecal valve, (*b*) caecum, colon, rectum and faeces.

(b) Distribution and elimination

The methods and doses used in investigating the concentrations in the cerebrospinal fluid of rabbits, the mode of urinary excretion in the hen and the tissue distribution in rats were the same as those used by Acred, Brown, Turner & Wright (1961). In all experiments the antibiotic was administered intramuscularly, but in the distribution experiments it was also given orally. The bile, blood and urine concentrations in conscious rats were determined using the method described above for intestinal administration, the antibiotic being given orally by stomach tube.

Protein binding

5 ml. of bovine, horse or human serum containing 5 mg of either ampicillin or phenoxymethyl penicillin was placed in cellophane bags (Visking Tubing ¾ in. diameter) and suspended in 20 ml. sterile saline at 10° C for 48 hr. At the end of the period of dialysis the amounts of the antibiotic outside and inside the tubing were assayed. Five tubes of each antibiotic were prepared in each experiment.

Chemotherapy

The protective effect of ampicillin was determined in mice infected with *Klebsiella pneumoniae, Salmonella typhimurium, Diplococcus pneumoniae, Streptococcus pyogenes* Group A, *Staphylococcus aureus* Smith (benzylpenicillin sensitive), and *Staphylococcus aureus* 52–75 (benzylpenicillin resistant, minimal inhibitory concentration 125 μg/ml.). The experimental infections were produced in groups of 10 mice by injections of the bacteria using from 100 to 500 median lethal doses of the bacterial culture, prepared in 5% hog gastric mucin to enhance the virulence (Table 1). The median lethal dose (LD50) was determined by

TABLE 1

THE MEDIAN LETHAL DOSE (MLD) OF BACTERIA AND THE MULTIPLE OF THIS DOSE ADMINISTERED TO INFECT THE ANIMALS IN THE PROTECTION TESTS (SEE TEXT)

Organism	Median lethal dose (LD50) organism/ml.	Infecting dose
Klebsiella pneumoniae	1×10^1	100 × MLD
Salmonella typhimurium	1×10^1	100 × MLD
Diplococcus pneumoniae	1×10^4	100 × MLD
Streptococcus pyogenes A	1×10^6	100 × MLD
Staphylococcus aureus Smith	1×10^2	500 × MLD
Staphylococcus aureus 52–75	1 in 2 dilution of an overnight broth culture with 5% hog gastric mucin	

preparing seven 10-fold dilutions of 18 to 25 hr cultures of the pathogens in 5% hog gastric mucin. 0.5 ml. of the dilutions was injected intraperitoneally into male albino mice (18 to 22 g), one dilution being administered to a group of 10. The animals were observed for 4 days and deaths were recorded each day. The median lethal dose was determined graphically by plotting the dilution factor of the culture against the percentage deaths, using log probit paper.

The median curative dose (CD50). Ampicillin, tetracycline, chloramphenicol, phenoxymethyl penicillin, benzylpenicillin and phenethicillin were administered subcutaneously and orally to the groups of mice. A 4:1 dose ratio was used, the doses being administered in 0.2 ml. normal saline immediately following the infection. The mice were observed for 4 days and the deaths were recorded daily. The percentage deaths were plotted against log dose on log probit paper, and the dose of compound (mg/kg) giving protection to 50% of the mice (CD50) was read off from the graph.

RESULTS

Toxicity

(i) *Acute.* Ampicillin is non-toxic to mice and rats when administered either orally or subcutaneously in doses of 5 g/kg.

As regards intravenous dosage for mice, 2 g/kg has been administered without lethal effects, although muscle tremors, slowed respiration and mild clonic convulsions have sometimes occurred. The amount which can be administered intravenously has been limited by solubility, and the maximum dose we have been able to give has been 2.5 g/kg. At this dose level 3 out of a group of 10 mice have died.

(ii) *Prolonged administration.* No toxic symptoms were noted in rats treated with ampicillin. Similarly there were no observable toxic symptoms in dogs apart from a slight loosening of the stools during the early days of the test. Post-mortem examination did not reveal any abnormalities in either species, and the histology of the organs examined was normal.

Local irritant and pharmacological action

No macroscopical signs of damage were observed apart from a slight area of erythema at the site of injection. Microscopical changes in the skin and muscle were minimal and difficult to find. They consisted only of very sparse inflammatory cell infiltration and slight accompanying interstitial fluid accumulation.

Doses up to 80 mg/kg, administered intravenously to cats, had no effect on blood pressure or respiration. Neither had they any effect on the blood pressure response to an injection of adrenaline, acetylcholine or histamine.

Absorption

(i) *Rabbits.* The blood levels obtained after oral and intramuscular administration of phenoxymethyl penicillin and ampicillin are given in Table 2. After intramuscular dosing the peak level of phenoxymethyl penicillin at 1 hr is higher than ampicillin, but at subsequent periods the concentrations of both antibiotics are similar. On the other hand, after oral administration the concentrations of ampicillin are considerably greater than phenoxymethyl penicillin.

TABLE 2

MEAN BLOOD CONCENTRATIONS FOLLOWING ORAL AND INTRAMUSCULAR ADMINISTRATION OF 100 MG/KG AMPICILLIN AND PHENOXYMETHYL PENICILLIN TO GROUPS OF 5 RABBITS

	Concentration μg/ml. of blood at hr				
Antibiotic	1	2	3	4	Route
Ampicillin	3·0	2·91	0·73	0·13	Intramuscular
Phenoxymethyl penicillin	4·7	2·42	0·65	0·22	
Ampicillin	2·16	0·92	0·27	0·06	Oral
Phenoxymethyl penicillin	0·67	0·5	0·03	0	

(ii) *Dogs.* The mean serum concentrations after 20 mg/kg of ampicillin and phenoxymethyl penicillin given orally and of 5 mg/kg ampicillin and benzylpenicillin given intramuscularly are shown in Table 3. Following the oral administration of phenoxymethyl penicillin, the highest blood concentration recorded occurred 30 min after administration, whereas with ampicillin the highest concentration occurred 1 hr afterwards. Throughout the 5 hr period of the test, when

TABLE 3

MEAN SERUM CONCENTRATIONS IN GROUPS OF 5 DOGS AFTER ORAL ADMINISTRATION OF 20 MG/KG AMPICILLIN AND PHENOXYMETHYL PENICILLIN AND INTRAMUSCULAR ADMINISTRATION OF 5 MG/KG AMPICILLIN AND BENZYLPENICILLIN

		Concentration μg/ml. at hr after dosing										
Antibiotic	Route	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0	5·5	24
Ampicillin	Oral	4·87	7·23	3·67	3·51	2·15	0·96	0·4	0·32	0·22	0·19	0·11	0
Phenoxymethyl penicillin	Oral	2·58	1·48	0·59	—	0·15	0·1	0·08	0·07	0·07	0·04	0·04	0
Ampicillin	Intramuscular	4·6	3·3	1·55	0·95	—	0·32	0·13	0·13	0·09	0·09		
Benzylpenicillin	Intramuscular	2·01	0·76	0·48	0·26	—	—	0·06	0·04	0·03	0·01		

blood samples were taken regularly, the concentration of ampicillin was always considerably higher than phenoxymethyl penicillin. After intramuscular administration the peak serum concentrations of ampicillin and benzylpenicillin occur at 30 min. Throughout the period of the test the concentrations of ampicillin were greater than benzylpenicillin.

(iii) *Rat—intestinal absorption.* The percentage of dose excreted in the bile and urine and that remaining in the gut after the 25 hr period of experimentation are shown in Table 4. Maximum biliary excretion occurred in the 2 to 4 hr period after dosing and there was no excretion in the bile after 23 hr. No urine samples were obtained during the first 2-hr period, and maximum urinary excretion occurred within 4 hr of dosing. Over the 25 hr period approximately twice as much ampicillin was excreted in the urine as compared with that excreted in the bile. In only one rat (no. 3) was there any appreciable quantity of ampicillin remaining in the small intestine after 25 hr. Similarly, the colon, caecum, rectum and faeces of only one rat contained a significant quantity of ampicillin after 25 hr. Approximately 7% of the dose could be accounted for in the bile, urine and faeces.

TABLE 4

ABSORPTION AND EXCRETION OF AMPICILLIN IN THE CONSCIOUS RAT AFTER INTRADUODENAL DOSING AT 100 MG/KG

[0* no sample obtained]

Rat no.	Dose mg	Bile. % of dose excreted (hr)					Urine. % of dose excreted (hr)					Blood levels. Concentration μg/ml. (hr)				% of dose remaining in gut after 25 hr	
		1–2	2–4	4–6	6–23	23–25	1–2	2–4	4–6	6–23	23–25	1	3	5	24	Small intestine	Colon, caecum, rectum, faeces
1	32	0·21	0·83	0·19	0·25	0	0*	0·53	0·04	0·63	0*	0·52	0·36	0·21	0	0·01	0·09
2	43	0·66	0·78	0·28	0·70	0	0*	0*	2·39	2·06	0·04	0·94	0·52	0·46	0	0	2·64
3	29	0·72	0·38	0·10	0·21	0	0*	1·32	0·35	0·84	0	1·35	0·56	0·28	0·11	2·71	0
4	30	0·16	0·71	0·18	0·06	0	0*	0·97	2·49	0·26	0	3·00	0·42	0·17	0	0	0
5	41	0·98	1·80	1·37	0·16	0	0*	3·76	0*	2·95	0·01	0·60	0·88	0·76	0	0·06	0
Mean		0·55	0·90	0·42	0·28	0	0*	1·65	1·32	1·35	0·01	1·28	0·55	0·38	0·02	0·56	0·55
Cumulative % excretion		0·55	1·45	1·87	2·15			1·65	2·97	4·28	4·29						

TABLE 5

MEAN CONCENTRATIONS IN BLOOD, URINE AND BILE, % OF DOSE EXCRETED IN URINE AND BILE, AND RATIO OF URINE AND BILE TO BLOOD CONCENTRATIONS AFTER ORAL ADMINISTRATION OF 100 MG/KG OF AMPICILLIN AND PHENOXYMETHYL PENICILLIN TO GROUPS OF 10 CONSCIOUS RATS

Time (hr)	Blood concentration μg/ml.		Urine concentration μg/ml.		Bile concentration μg/ml.		% of dose excreted in urine		% of dose excreted in bile		Ratio of urine to blood concentration		Ratio of bile to blood concentration	
	Ampicillin	Phenoxymethyl penicillin	Ampicillin	Phenoxymethyl penicillin	Ampicillin	Phenoxymethyl penicillin	Ampicillin	Phenoxymethyl penicillin	Ampicillin	Phenoxymethyl penicillin	Ampicillin	Phenoxymethyl penicillin	Ampicillin	Phenoxymethyl penicillin
0–2	0·25	1·42	171·4	546·5	65·9	441·0	0·69	3·55	0·27	3·05	690	385	265	311
2–4	0·41	1·27	234·8	384·6	95·3	503·6	1·46	3·90	0·65	4·19	569	303	231	397
4–6	0·19	0·41	235·2	225·8	83·7	96·6	1·08	0·66	0·41	0·55	1,238	551	441	236

Distribution

Cerebrospinal fluid. Ampicillin penetrates into the cerebrospinal fluid with difficulty. The concentrations in the cerebrospinal fluid after doses of 500 mg/kg intramuscularly at 1, 2, 4 and 6 hr following administration are respectively 1.4, 0.12, 0.05 and 0.13 μg/ml., whereas the corresponding levels in the serum are 54.0, 14.25, 4.2 and 5.8 μg/ml.

Elimination in the conscious rat. Table 5 shows the mean blood, urine and bile concentrations of phenoxymethyl penicillin and ampicillin at 2-hr intervals following the administration of 100 mg/kg orally of each antibiotic. The concentration ratio from the blood to the urine, and from the blood to the bile, is also given. The mean ratios show that there is little difference between the two antibiotics regarding the blood/bile concentration, the bile concentration being approximately 300 times that found in the blood. However, the concentration of ampicillin in the urine is twice that of phenoxymethyl penicillin, and the mean blood to urine concentration ratios of each antibiotic are 800 for ampicillin and 400 for phenoxymethyl penicillin.

Urinary excretion—the hen. In hens treated with ampicillin only, almost all of the antibiotic administered is excreted in the urine during the 6-hr period following intramuscular administration into the left leg. Four to five times more is excreted by the left kidney than by the right kidney. After probenecid which blocks renal tubular filtration the mean total amount excreted by the left kidney is reduced to about 1.6 times that excreted by the right kidney ; moreover, following probenecid approximately 66% of the injected dose only can be accounted for during the period of test (see Table 6).

TABLE 6

EXCRETION OF AMPICILLIN IN THE HEN

The mean urinary excretion from the left and right kidneys of 4 hens each of which received 100 mg ampicillin and 100 mg ampicillin + 100 mg probenecid. Ampicillin was administered into the left leg muscles and probenecid into a wing vein

Treatment	Time (hr)	% of dose excreted	
		Left	Right
Ampicillin	0–1	36·78	6·05
	1–2	19·10	4·49
	2–3	9·07	1·19
	3–4	7·34	1·40
	4–5	3·85	1·56
	5–6	1·64	1·99
	Total 0–6	77·78	16·68
Ampicillin with probenecid	0–1	18·56	12·75
	1–2	10·07	6·85
	2–3	5·51	1·99
	3–4	3·52	1·41
	4–5	1·93	1·87
	5–6	1·16	0·85
	Total 0–6	40·75	25·72

Tissue distribution. The amounts of ampicillin recovered from the various tissues after oral and intramuscular administration are shown in Tables 7 and 8. The amounts are expressed in μg/g wet weight (column a) and as the ratio of concentration in the tissues to the concentration in the serum (column b). The values

TABLE 7

DISTRIBUTION OF AMPICILLIN IN RATS AFTER ORAL ADMINISTRATION

Seven groups of 10 rats were given orally 100 mg/kg ampicillin. One group was killed at the end of each time period. The mean concentration of ampicillin is expressed in μg/g wet weight of tissue (column a) and the concentration ratio between the tissues μg/g wet weight to serum μg/ml. is shown in column b. The urinary excretion is expressed as a % of the dose administered

a = concentration μg/ml. b = $\frac{\text{concentration in tissue}}{\text{concentration in serum}}$ * = % recovered from whole carcass

		0·5 hr		1 hr		2 hr		4 hr		12 hr		24 hr	
Organ	0 hr	a	b	a	b	a	b	a	b	a	b	a	b
Liver		15·82	3·58	18·0	9·18	8·16	9·27	2·17	27·13	0·08	6·4	0	—
Spleen		2·22	0·5	0·39	0·2	0·33	0·38	0·12	1·5	0·03	2·64	0·05	—
Kidneys		13·22	2·99	9·86	5·03	6·31	7·17	1·04	13·0	0·06	5·0	0	—
Lungs		6·17	1·4	1·72	0·88	1·29	1·47	1·08	13·5	0·03	1·8	0·08	—
Stomach		706·0	159·7	372·4	190·0	198·0	225·0	5·65	70·63	8·75	700·0	0·09	—
Small intestine		1,230·0	278·3	1,260·0	642·9	666·0	756·8	37·66	470·7	1·15	92·0	0·13	—
Caecum, colon		2·41	0·55	0·35	0·18	531·9	604·5	1,482·0	18,525·0	73·5	5,880·0	2·77	—
Faeces		0	0	0	0	0·104	0·12	660·8	8,260·0	26·18	2,094·0	77·5	—
Carcass		3·57	0·81	0·71	0·36	1·95	2·22	0·68	8·5	0·125	10·0	0	—
Serum		4·42	1·0	1·96	1·0	0·88	1·0	0·88	1·0	0·012	1·0	0	—
% urine excreted		0·2		0·87		3·11		4·34		6·63		3·7	
% dose recovered	*60·38	64·8		57·9		45·03		42·65		9·61		4·31	

TABLE 8

DISTRIBUTION OF AMPICILLIN GIVEN INTRAMUSCULARLY, IN RATS

Five groups of 10 rats were injected intramuscularly with 100 mg/kg ampicillin. One group was killed at the end of each time period. The mean concentration of ampicillin is expressed in μg/g wet weight of tissue (column a) and the concentration ratio between the tissues μg/g wet weight to serum μg/ml. is shown in column b. The urinary excretion is expressed as a % of the dose administered.

$$a = \text{concentration } \mu g/ml. \qquad b = \frac{\text{concentration in tissue}}{\text{concentration in serum}}$$

Organ	0 hr		0·5 hr		1 hr		2 hr		4 hr	
	a	b	a	b	a	b	a	b	a	b
Liver			175·0	2·7	70·48	4·6	5·76	6·1	0·98	5·4
Spleen			8·3	0·13	3·4	0·22	0·76	0·8	0·24	1·3
Kidneys			288·0	4·4	146·2	9·6	6·56	6·9	0·75	4·2
Lungs			28·6	0·44	6·7	0·44	1·18	1·3	0·52	2·9
Small intestine			77·4	1·2	111·7	7·3	70·8	75·3	15·56	86·4
Large intestine			6·74	0·1	5·24	0·34	20·1	21·4	105·8	587·7
Site of injection	1,700·0	204·6	132·0	2·01	31·4	2·1	3·86	41·1	1·86	10·3
Carcass	2·64	0·32	17·9	0·28	4·54	0·3	0·78	0·8	0·85	4·7
Urine			2,548·0	3·9	4,105·0	268·7	2,626·0	2,793·6	1,452·0	8,066·6
Serum	8·31	1·0	64·4	1·0	15·28	1·0	0·94	1·0	0·18	1·0
Faeces							13·0	13·8	23·32	129·5
% recovered in urine	—		52·0		84·4		112·0		95·5	
% dose recovered	85·6		73·2		99·4		116·9		99·9	

represent the mean of 10 rats. The amount of the antibiotic recovered expressed as a percentage of the dose administered is shown in the tables.

After oral administration the concentration of ampicillin fell off more rapidly in the serum than any other tissue, with the exception of the spleen. The high concentrations in the livers and kidneys were probably accounted for by the presence of bile and urine in these organs. The passage of the antibiotic along the alimentary canal is well illustrated. The antibiotic passed very rapidly from the stomach to the small intestine. There were only trace amounts found in the colon for the first 2 hr of the test, but at 4 hr large quantities were recovered in the colon and faeces. Throughout the period of the test the total quantity recovered decreased until at 12 and 24 hr only 9.6% and 4.3% of the doses administered were recoverable ; the majority of these quantities were in the urine, 6.6% and 3.7% respectively. After oral administration 43 to 65% can be recovered in the first 4 hr.

After intramuscular administration there is a rapid increase of serum level during the first half-hour following administration ; however, this is followed by a very rapid decrease in serum levels. Within 4 hr serum levels have been reduced from 64.4 μg/ml. to 0.18 μg/ml. Practically the whole of the amount injected is recovered in the urine within 1 to 2 hr after administration. The concentrations occurring in the liver and the kidneys are also initially very high, and they decline very rapidly but not quite at the same speed as the serum levels. The concentrations in the spleen and lung during the first hour never reach the concentration found in the serum, but the decline in the concentrations in these two organs is much less than in the serum, so that at the end of 4 hr the concentrations are higher than the serum. The amount found in the small intestine and the large intestine resulting probably from biliary excretion does not exceed 6.5% of the dose administered. After intramuscular injection 73 to 117% can be recovered in the first 4 hr.

Protein binding

Ampicillin is considerably less bound to serum than phenoxymethylpenicillin (Table 9).

TABLE 9

PROTEIN BINDING

Mean % of ampicillin and phenoxymethyl penicillin bound to bovine, horse and human serum

	Mean % serum bound		
Antibiotic	Bovine	Horse	Human
Ampicillin	17·15	7·9	17·0
Phenoxymethyl penicillin	51·37	39·37	68·7

Chemotherapy

Ampicillin is effective against a number of experimental infections produced by gram-negative and gram-positive organisms in mice (Table 10). Against the penicillin-sensitive staphylococci (*Staphylococcus aureus* Smith) ampicillin, benzylpenicillin and phenethicillin, given subcutaneously, are the most active antibiotics followed by phenoxymethyl penicillin and tetracycline ; chloramphenicol is inactive.

TABLE 10

ACTIVITY OF AMPICILLIN, PHENOXYMETHYL PENICILLIN, PHENETHICILLIN, BENZYLPENICILLIN, TETRACYCLINE AND CHLORAMPHENICOL AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE INFECTIONS

Expressed in terms of the dose of antibiotic calculated to protect 50% of a group of infected mice (CD50 mg/kg)

n=number of observations

Organism	Antibiotic	*In vitro* M.I.C. μg/ml.	Route–Oral Mean CD50 mg/kg	n	Route–Subcutaneous Mean CD50 mg/kg	n
Staphylococcus aureus Smith	Ampicillin	0·1	0·3	4	0·3	4
	Phenoxymethyl penicillin	0·02	0·3	3	0·5	1
	Benzylpenicillin	0·02	5·8	1	0·3	3
	Phenethicillin	0·05	0·3	2	0·1	1
	Tetracycline	0·1	5·2	1	6·0	1
	Chloramphenicol	50·0	Inactive 100	2	Inactive 100	2
Staphylococcus aureus 52–75	Ampicillin	>200·0	Inactive		Inactive	
	Phenoxymethyl penicillin	>200·0	Inactive		Inactive	
	Benzylpenicillin	>200·0	Inactive		Inactive	
	Phenethicillin	>200·0	Inactive		Inactive	
	Tetracycline	>200·0	Inactive		Inactive	
	Chloramphenicol	>200·0	Inactive		Inactive	
Streptococcus pyogenes Group A	Ampicillin	0·1	0·1	1	0·025	
	Phenoxymethyl penicillin	0·05	0·1	1	0·1	1
	Benzylpenicillin	0·05	—		—	
	Phenethicillin	0·2	0·5	1	0·1	1
	Tetracycline	0·2	0·5	1	0·5	1
	Chloramphenicol	2·0	3·2	1	3·2	1
Diplococcus pneumoniae	Ampicillin	0·05	0·25	2	0·5	2
	Phenoxymethyl penicillin	0·05	0·6	2	0·9	2
	Benzylpenicillin	0·02	—	2	0·6	2
	Phenethicillin	0·05	0·4	1	0·2	1
	Tetracycline	0·2	13·0	1	5·0	2
	Chloramphenicol	2·0	>100·0	1	Inactive 100	1
Klebsiella pneumoniae	Ampicillin	0·5	11·6	9	35·4	8
	Phenoxymethyl penicillin	50·0	Inactive 400	4	Inactive 400	4
	Benzylpenicillin	10·0	—		Inactive 400	3
	Phenethicillin	500·0	Inactive 400	2	Inactive 400	2
	Tetracycline	0·5	Inactive 400		61·0	14
	Chloramphenicol	1·0	165·0		280·0	2
Salmonella typhimurium	Ampicillin	1·0	18·0	10	12·8	10
	Phenoxymethyl penicillin	200·0	Inactive 400	4	Inactive 400	4
	Benzylpenicillin	10·0	Inactive 400	3	82·0	3
	Phenethicillin	>100·0	Inactive 400	2	Inactive 400	2
	Tetracycline	2·0	62·4	5	59·2	16
	Chloramphenicol	5·0	310·0	1	250·0	3

When given by the oral route ampicillin, phenoxymethyl penicillin and phenethicillin are equally active, but the activity of benzylpenicillin is reduced to that of tetracycline ; chloramphenicol again is inactive. None of the antibiotics is active against staphylococci resistant to benzylpenicillin.

Against streptococci, ampicillin, phenoxymethyl penicillin, phenethicillin and tetracycline are all very active when given orally or subcutaneously, although

ampicillin is the most active when given subcutaneously. Again, chloramphenicol is the least active.

Against pneumococci, ampicillin is the most active, followed by phenoxymethyl penicillin, phenethicillin and benzylpenicillin. Tetracycline is less active and chloramphenicol is only slightly active.

In the animals infected with gram-negative organisms, benzylpenicillin, phenethicillin and phenoxymethyl penicillin are inactive, while ampicillin is considerably more active than tetracycline, which in turn is more active than chloramphenicol, both orally and subcutaneously.

DISCUSSION

Ampicillin is the first semi-synthetic penicillin which is effective orally against gram-negative and gram-positive organisms. Unlike other broad-spectrum antibiotics, which are usually limited in their usefulness by untoward side-effects, ampicillin is non-toxic. As with benzylpenicillin, high doses can be administered safely by injection and by mouth to laboratory animals. In the prolonged toxicity studies the only observable symptoms occurred in dogs, where the initial dosing resulted in the passage of semi-liquid stools. In contrast to tetracycline and chloramphenicol, which produce severe tissue damage at the sites of injection (Hanson, 1961), ampicillin after intramuscular and intradermal administration has little irritant effects.

In the rat after oral and intraduodenal administration, ampicillin and phenoxymethyl penicillin are poorly absorbed. However, in dogs after oral administration, ampicillin gives higher blood levels than phenoxymethyl penicillin. The levels are also higher than those obtained after administration of much larger doses to rats. In humans, ampicillin (Knudsen, Rolinson & Stevens, 1961) also gives good blood levels after oral administration ; and 30% of the antibiotic can be recovered in the urine within 6 hr. The results indicate that the rat is an unsuitable animal for the study of oral absorption of penicillin derivatives.

After oral administration a large proportion of the antibiotic is destroyed in the intestine. Since the antibiotic is not stable to bacterial " penicillinase " it can be assumed that it is destroyed by the intestinal flora. There would appear to be no destruction of the antibiotic within the body, as bile and urine chromatography revealed only unchanged ampicillin.

After intramuscular administration almost all of the antibiotic is recovered in the urine in 2 hr. This is in marked contrast to the oral results, where the total recovery in the urine does not exceed 10%. The concentration of ampicillin in the bile and urine during the first 4 hr after administration to rats is 600 and 250 times respectively the corresponding serum concentrations, these concentration ratios being similar to those found with methicillin. However, in the experiments in which the bile ducts were cannulated, it would appear that the stress of the operation reduces the urinary excretion considerably, since only 23.4% of the antibiotic is recovered in 4 hr, whereas 95% of the antibiotic is recovered in the urine in the unoperated animals within the same time period.

Ampicillin is rapidly cleared from the blood by the kidneys. It is removed both by renal tubular secretion and glomerular filtration. This was demonstrated in the hen, which possesses a renal portal system (Sperber, 1949). The venous return from the hind limbs is shunted through the parenchyma of the renal tubules. Therefore, when a substance is injected into a hind limb and both renal tubular secretion and glomerular filtration take place, the concentration of the substance excreted is greater in the urine from the ipsilateral kidney than in the urine from the contralateral kidney. If only glomerular filtration takes place, then the concentration in the urine excreted from both kidneys is identical. In our experiments, prior to the block in tubular secretion by probenecid, 95% of the dose of ampicillin administered is excreted within 6 hr following administration, and of this 78% is excreted by the kidney nearest to the injection site, while only 16.6% is excreted by the contralateral kidney. On the other hand, after the administration of probenecid, although the amount excreted by the contralateral kidney is increased and that excreted by the other kidney is reduced, the kidneys still do not excrete equal amounts, suggesting incomplete block of renal tubular secretion. Knudsen (personal communication) has found that in humans a single dose of probenecid similarly does not block completely renal tubular secretion of ampicillin. A second dose is required to produce complete block.

Ampicillin is distributed throughout the body tissues, but is concentrated only in the kidney and liver, the high values in these organs probably being attributed to the presence of urine and bile respectively. As with other penicillins, only small quantities penetrate the cerebrospinal fluid.

Benzylpenicillin, phenoxymethyl penicillin, phenethicillin and ampicillin all have the same order of activity *in vitro* against the gram-positive organisms, and this relationship in activity is born out *in vivo*. Tetracycline likewise gives a reasonable parallelism between *in vivo* and *in vitro* activity. On the other hand, chloramphenicol has a CD50 of 3.2 mg/kg against the streptococcal infection, but is inactive in doses of 100 mg/kg against *Staphylococcus aureus* Smith and *Diplococcus pneumoniae*, although *in vitro* the activity of chloramphenicol against the three organisms differs by only one tube dilution.

Against the gram-negative organisms which were investigated the activity of ampicillin *in vivo* corresponds to its *in vitro* activity, but both tetracycline and chloramphenicol are considerably less active *in vivo* than ampicillin, even though their *in vitro* activities are only slightly less than ampicillin.

There is no valid explanation for these differences at present. They cannot be attributed simply to ampicillin being better absorbed, being less protein-bound or being distributed throughout the body more effectively than either tetracycline or chloramphenicol, since if this were so it would be expected that the difference in the *in vivo* and *in vitro* activities of these antibiotics would be the same against the gram-positive as well as the gram-negative organisms. In the assessment of the activity of chemotherapeutic agents, therefore, these results emphasize the need to correlate all the *in vitro* and *in vivo* experimental results.

We wish to thank Dr A. C. Thackray, of the Bland Sutton Institute of Pathology, for expert histological comment; Dr A. A. G. Lewis, Physician to the Connaught Hospital,

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CONFIDENTIAL

PHARMACOLOGY OF PENICILLINS

by

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A great deal has been written about the significance of protein binding of antibiotics and it is commonly accepted that if the antibiotic is bound to the body proteins only the free antibiotic is active. In other words an antibiotic which is bound to the extent of 90% to serum albumin will only show activity in the serum equal to one tenth of the total concentration of antibiotic tested in the absence of serum albumin. This concept developed by pharmacologists in other spheres appears to be rational and taken as a whole would seem to be satisfactory. However the systems which have been studied have in general been in vitro and the conditions hardly approximate to those found in vivo where there is a continuing changing environment. The rate of absorption, elimination and destruction of the drug is not acccunted for in vitro; neither do in vitro systems take into account natural anatomical barriers or barriers produced as a result of bacterial infection or inflammatory lesions. Hence the effect of an antibiotic cannot be fully predicted by means of in vitro tests. Infections are rarely systemic and are in general confined to specific sites. The activity of the antibiotic in the blood while of considerable importance is not of over-riding importance and it is perhaps of much greater interest to determine the concentration of antibiotic at the site of infection. The effectiveness of an antibiotic in vivo can also be determined by assessing the effectiveness of an antibiotic against localised infections. Only a limited number of attempts have been made to study the action of antibiotics against localised infections. Eagle, Fleischman and Musselmann (1950) examined the effect of penicillin in mice which had received an intramuscular injection of pneumococci and streptococci and in rabbits which had intratesticular injection. Selbie studied the development of the staphylococcus after intramuscular injection in mice and Selbie and O'Grady (1954) examined tuberculous lesions similarly produced.

The methods largely depend on the selection of the correct strain of organism for the species used and unless an adequately virulent infection can be established the tests are difficult to interpret and can be misleading. By selection of the appropriate strain of mouse and staphylococcus Brown and Acred (1960) developed the thigh lesion technique of Selbie and have screened a large number of semi-synthetic penicillins. A comparision of activity in vivo of penicillins having different protein binding properties can now be made. The results obtained in mice with a number of penicillins are shown in Table I but it must be understood that the protein binding figures are those determined in human serum. The actual binding in mouse serum could be less but it is not unreasonable to assume that the ratio of binding is the same. The results illustrate that irrespective of variations in binding the activities of the antibiotics are of the same order. This suggests two alternatives a) the bound antibiotic is active and penetrates to the site of infection b) the amount of free penicillin present in the infection is equal for each antibiotic and that this free concentration is not the same as in the blood. The latter explanation is more likely but the precise reasons for this have not been elucidated.

CD_{50} tests which depend on administering a lethal infection to a group of animals then determining the amount of antibiotic which will protect 50% of the group also provide information on the value of an antibiotic in vivo. The animals are infected intraperitoneally and the drugs are given subcutaneously. To be effective therefore they must be taken up on the blood and then penetrate into the peritoneal fluids. Our results (Table II) obtained with CD_{50} tests against staphylococci illustrates another facet in the problem. The protein binding figures obtained by static dialysis technique show that the antibiotics tested are all fairly heavily bound and more or less within a given range. However, when the binding was determined by continuous dialysis over a period of 24 hours two groups clearly emerge; one where the antibiotic is nearly all removed from the serum and the second group where there is still a significant quantity of the antibiotic retained.

TABLE I.

Antibiotic	Thigh Lesion % Protection SO		Protein Binding Human
	200 mg/kg	50 mg/kg	Static Dialysis
1400	97.2	86.9	45
1577	97.9	85.7	67
1621	98.8	87	71
1702	97.8	91.3	90
1454	87.3	60.6	75
1751	98.9	86.6	50

PENICILLIN	PROTEIN BINDING PERCENTAGE		CD_{50} mg/kg
	STATIC	CONTINUOUS	
BROXIL	86	17.5	0.3
AB 1929	75	20	0.1
AB 1936	65	3	0.3
AB 1904	92	15	0.3
AB 2001	66	56	1.8
AB 2006	97	43	1.8
AB 2011	96	54	1.2
AB 2033	95	48	1.3

TABLE II

Protein binding and activity of a number of penicillins of the "Broxil" series and their activity against Staphylococcus aureus infection in mice.

The antibiotics of the first group will give CD_{50} values of a low order i.e. they are all very active, and those in the second group give much less satisfactory CD_{50} values.

Huang, Seto, Weaver, English, McBride and Shull (1963) comment on their studies with α-substituted penicillin amides that in spite of low in vitro activity and mediocre serum levels that α-methyoxy-n-propyl penicillinamide is an extremely effective chemotherapeutic agent. From their observations they state that in vitro activity and serum levels are by no means the only decisive factors which determine chemotherapeutic efficacy of an antibiotic.

The results of both Acred and Brown and Huang et al could be criticised on the grounds that the activities in the animal could differ because of differing degrees of protein binding between the serum of animal species under test and human serum. However particularly with regard to the results of the first investigators it is perhaps more than co-incidence that the results have separated out in the way they have done.

Penetration of Penicillin into extravascular sites

In the early days of penicillin therapy there was a strong belief that the concentration of penicillin in the blood was insufficient to account for its antibacterial action. Jawetz (1946) and White, Lee and Alveson (1946) showed in animal experiments that the duration of survival depends more on the total dose of antibiotic given rather than the interval between injections. To explain the observations and to study further the fate of penicillin in the body with a view to predicting its action many workers have attempted to measure the active concentration of penicillin in tissues. Florey, Chain and their associates (1941) were the first to report on the distribution of penicillin in experimental animals after administration by various routes. They found that high concentration appeared in the urine and that it was also present in saliva, although they could

demonstrate none in tears or pancreatic juice. M.E. Florey (1946) measured the amount of penicillin in human wound exudates after intramuscular administration of 100,000 units (= 66 mg). The antibiotic was found in every case after 3 hours and in 50% of the cases it was detectable 12 hours afterwards while the concentration in the blood fell rapidly to zero after 4 hours. At 4 hours the blood concentration was 0.004 - 0.08 units/ml and the exudate concentrations were 0.16 - 0.32 units/ml. This work is supported by Weinstein, Daikos and Perrin (1951). They placed sterile fibrin clots in several areas under the rabbit skin on the back. After the intramuscular administration of various doses of penicillin the clots were removed and assayed. They found that in the first hour the blood levels were more than in the clots but subsequently the levels were higher on the clots and activity was still detectable twelve hours afterwards. Applying the same principle as Weinstein et al, Werner, Knight and McDermott (1954) implanted specially prepared agar discs in the peritoneal cavity of rabbits. The animals were given a large intravenous dose of the penicillin and streptomycin and the discs were removed for assay at intervals. Blood was taken from the cardiac vein or by cardiac puncture. High concentrations were readily attained in the agar discs and concentrations equal to or exceeding the serum concentration were achieved in 1 - 2 hours in the case of penicillin and 1 - 4 hours with streptomycin. In a second series of tests with cats a comparision was made with chloramphenicol and penicillin and they found that the levels of chloramphenicol did not achieve the same magnitude as found with penicillin. Nathanson and Liebhold (1946) found that sulphonamides did not penetrate into fibrin clots but penicillin was freely diffusible. McCune (1960) extended the work of Werner et al and implanted agar discs in the peritoneal cavity of rabbits. He allowed 28 days for a fibrous capsule to be produced round the disc then he injected penicillin intravenously. At given intervals the discs were removed from the rabbit and the penicillin concentration estimated. Within two hours of administration concentrations of penicillin in the disc were much higher than in the blood but on the other hand in a comparable test with streptomycin the levels in the disc were no higher than the blood. Using implanted cotton pellets to produce a granuloma Brown (1964) found that the concentrations of four semi-synthetic penicillins found in the granulomas were equal to the concentrations found in the blood. In order to obtain

further information on the concentration of penicillins in inflammatory fluids Ungar (1950) measured the amount appearing in inflammatory fluids following intramuscular injection of 0.5 ml turpentine. When sufficient oedema had been produced 24 hours later the penicillin was given intramuscularly and the fluid and blood levels were determined at intervals afterwards. Within one to two hours the levels in the exudates were higher than the blood levels and persisted much longer. Brown (1964) found that following the intraperitoneal injection of 0.1 ml of turpentine to rats that the concentrations of Cloxacillin, Phenethicillin and Penicillin V were slightly higher than the corresponding blood levels while the concentration of Ampicillin in the inflammatory fluid was considerably higher. A comparision of Quinacillin and Methicillin by Hale and Spooner (1964) where the concentrations were determined in pus in an abscess produced by staphylococci in rats showed that both the penicillins gave persistent levels in the pus over those found in the blood. The experimental evidence that penicillin persisted in tissues long after its disappearance from the blood led Schacter (1948) to examine the concentration of penicillin appearing in the lymphatic system. Dogs were suitably anaesthetised and the thoracic lymph duct cannulated. Penicillin was injected intravenously and intramuscularly and samples of lymph and blood taken from the femoral vein were removed for assay at intervals. The tests showed that the penicillin lymph levels were more prolonged than the blood levels and after two hours the concentrations in the lymph were higher than the blood. Vervey and Williams (1962) examined a number of penicillins with a view to determining the penetration from plasma into lymph. They cannulated the peripheral lymphatic ducts in dogs and after a primary dose of the penicillin continued to administer the penicillin by slow intravenous infusion. Samples of lymph and blood were taken at intervals throughout the test. They found that apart from Methicillin none of the other penicillins gave as high levels in the lymph as in the blood. In a series of experiments with rats, Brown (1964) and Acred, Brown and Clarke (1964) found that thoracic lymphatic concentrations were generally higher than those found in the blood after intramuscular administration. Ampicillin and Penicillin G gave

values in the lymph which were twice the serum concentrations. In preliminary experiments in dogs Acred and Clarke (1964) have collected the peripheral lymph and compared the concentrations in the lymph with the blood taken from the right and left femoral veins after intramuscular administration into the left leg. The results indicated that after initial levels in the blood higher than in the lymph, the lymph concentrations continue to rise steadily, and within 2 - 3 hours are in excess of the blood concentrations. Peak levels have so far not been obtained in the lymph.

Apart from the work of Acred and Brown (1963); Acred, Brown, Turner, and Wright (1961); Acred, Brown, Turner and Wilson (1962) no systemic investigation has been carried out with penicillins to determine the mode of excretion of penicillin and to obtain information on the amount excreted in a given period. Acred and Brown used the hen which has a renal portal system and injected intramuscularly into the left leg the antibiotic under test. The urine was collected directly from the ureters at intervals. The total quantity of penicillin excreted by the right and left kidneys was recorded. The slopes of the elimination curves were all identical apart from the experiment with Ampicillin. The excretion of Ampicillin from the right kidney in the chicken which did not receive probenecid was extremely low. The bulk of the antibiotic was excreted by the left kidney which received the blood draining the injection site and therefore contained a higher concentration of antibiotic than the blood in the other leg. After the administration of probenecid the slopes for the excretion of Ampicillin from the right and left kidneys became similar and corresponded to the excretion eliminated in one hour. Table III gives the percentage of the total dose administered, by the right and left kidneys before and after the administration of probenecid.

The results indicate that the amount excreted by glomerular filtration for all the penicillins tested are very similar. Ampicillin would appear to be excreted largely by this route. Taking into consideration therefore all the experimental results, it is important to decide in which way protein binding affects the activity of antibiotic particularly penicillin.

	R	L	R	L
Ampicillin	16.7	77.9	25.9	40.8
Penicillin G	14.1	38.3	25.3	28.5
Methicillin	19.7	37.7	20.2	23.0
Cloxacillin	31.0	38.4	18.0	18.0

TABLE III

Percentage of Penicillin eliminated by the right and left kidneys of the hen before and after the administration of Probenecid.

When the concentration of a penicillin is measured in body fluids information must be available on the degree of binding in these fluids. The technical difficulties of such an exercise is considerable. Scholton (1963) has attempted to determine the concentration of antibiotic present in tissue fluids but since his methods depend on macerating the tissue and spinning down the solids, it is more than likely that he is measuring largely the concentration in the serum. Vervey and Williams (1962) assayed the concentration in the lymph following slow intravenous infusion of the penicillin. They measured total levels, their assay procedure was the usual cup plate technique using Sarcina lutea as the test organism. Specimens were diluted in buffer, 1 in 10 for lymph, prior to assay. The total amount bound in plasma and in lymph for a number of antibiotics was measured by ultrafiltration techniques. Their results indicated that "although the proteins in lymph are predominantly derived from plasma their affinity for penicillin is decreased". They could only hazzard a guess at the reason. Table 4 presents the "free" penicillin in plasma and peripheral lymph as a percentage of total penicillin in plasma. If these figures are correct they indicate that a higher percentage of free penicillin exists in the tissues than in the blood. This therefore extends the actual usefulness of an antibiotic. In addition if Brown and Acred's result also reflects a true state of affairs higher concentrations of antibiotic can be present in the extravascular fluids and coupled with Vervey and Williams' figures a still higher free level of antibiotic would be present. The report Brown and Acred, also shows that the free concentration of penicillin in the lymph is higher than in the serum. The results were obtained by ultrafiltration of rat lymph and serum.

These experimental results therefore imply that blood levels and the degree of binding in the blood are not the only factor in assessing the value of an antibiotic in vivo. It is possible that higher concentrations of total and free penicillin can exist in the extravascular fluids. On the surface this would appear to contradict the simple model of reversible diffusion of the free antibiotic across a semi-permeable membrane leading to a build up of antibiotic in the extravascular spaces depending on the protein available for binding.

TABLE IV

Antibiotic	Plasma	Lymph
Methicillin	79	90
Penicillin G	56	61
Phenethicillin	39	47
Penicillin V	36	49
Oxacillin	35	32
SKF 12141	23	31
PA 253	19	30

The "free" penicillin in plasma and peripheral lymph as a percentage of total penicillin in plasma.

The concept implies that the "free" level of antibiotic throughout the body is constant. Both Vesey and Williams' and Brown and Acreds' results suggest some alternative, and this may be provided by the work of Malek, Herold, Hoffman and Kolc (1957). These investigators have combined stretpomycin, neomycin and viomycin and streptomycin with carboxyl sulphonic or phosphorylated high molecular compounds and studied changes in their pharmacological properties. They found in dogs that the preparations referred to as Antibiolymphins appeared in the lymph in much higher concentration than in the blood. Penicillin as such was not studied but the mechanisms whereby the antibiotic preparations were concentrated in the lymph relative to the blood, could similarly apply with penicillins. Ampicillin for example could be carried through into the lymph in combination with a macromolecule and concentrated within the lymphatic system. Whether the product is active would have to be determined. However if one assumes that it is, an explanation for the enhanced activity of Ampicillin in in vivo experiments in relation to chloramphenicol would be forthcoming.

DMB/PS

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CHEMISTRY, TOXICOLOGY, PHARMACOLOGY AND MICRO-BIOLOGY OF A NEW ACID-STABLE PENICILLIN, RESISTANT TO PENICILLINASE *(BRL.1621)*

By

J. H. C. NAYLER, A. A. W. LONG, D. M. BROWN, P. ACRED, G. N. ROLINSON, F. R. BATCHELOR, SHIRLEY STEVENS and R. SUTHERLAND

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CHEMISTRY, TOXICOLOGY, PHARMACOLOGY AND MICROBIOLOGY OF A NEW ACID-STABLE PENICILLIN, RESISTANT TO PENICILLINASE *(BRL.1621*)*

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THE use of methicillin, 2,6-dimethoxyphenyl, penicillin, in the treatment of infections caused by penicillinase-producing strains of staphylococci is now well established in clinical practice. Its one disadvantage is that it must be given by injection since it is unstable in acid solution and poorly absorbed from the gastrointestinal tract. This article describes a new penicillin which does not suffer from this defect, and is, moreover, active at lower concentrations than methicillin. Clinical investigations are being reported concurrently elsewhere[1].

Chemistry

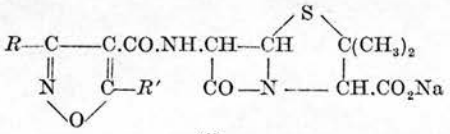
Acylation of 6-aminopenicillanic acid with reactive derivatives of several types of sterically hindered carboxylic acid gives penicillins which resist inactivation by penicillinase. These penicillins vary considerably in their chemical and antibacterial properties, and a particular class which combines resistance to both penicillinase and mineral acid with useful activity against most Gram-positive bacteria consists of 3,5-disubstituted 4-isoxazolyl penicillins (I). The preparation of two such penicillins, *BRL.*1400† ('Oxacillin') [I; $R = C_6H_5$, $R' = CH_3$] and its isomer, *BRL.*1454 [I; $R = CH_3$, $R' = C_6H_5$], has

* 'Orbenin', Beecham Research Laboratories, Limited, registered trade mark.

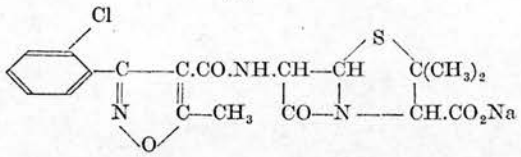
† 'Prostaphlin', Bristol Laboratories, Inc., registered trade mark.

been described in detail[2]. Many other penicillins of type (I) have been prepared[3] and examined, and one of them, 3-*o* chlorophenyl-5-methyl-4-isoxazolyl penicillin [II; *BRL*.1621], has been selected as the compound of choice.

BRL.1621 is available as the sodium salt monohydrate, which forms a colourless microcrystalline



(I)



(II) *BRL.1621**

powder. It is freely soluble in cold water to give a solution stable at 5° C for one week. A 1 per cent solution in water has $[\alpha]_D^{20}$ + 163° and *p*H of 6·0–7·5. The half-life in solution in 50 per cent aqueous alcohol at *p*H 1·3 and 35° C as determined by a previously described method[4] is 160 min, which is approximately equal to that of *BRL*.1400 and penicillin V.

Animal Studies, Toxicology and Pharmacology

Protection tests. The protective effect of *BRL*.1621 against the penicillin-resistant *Staphylococcus pyogenes* Russell (type 80) was estimated using the method of Selbie and O'Grady[5] as modified by Brown and Acred[6]. Groups of 10 mice (18–22 g) were infected by injecting into the muscle sheath of the thigh 0·2 ml. of a 1/2 dilution of an overnight growth of *Staphylococcus pyogenes* Russell. *BRL*.1621, methicillin and penicillin *G* were administered in doses of 200 and 50 mg/kg orally and subcutaneously, daily for three days, the first injection being given immediately following the infection. An infected control group received no treatment. The maximum thigh diameter of each mouse was measured with calipers on the first, second, third and sixth day after infection. The results are expressed as the percentage protection calculated in the following manner.

$$\frac{\text{mean daily thigh enlargement infected controls} - \text{mean daily thigh enlargement penicillin group}}{\text{mean daily thigh enlargement of infected controls}} \times \frac{100}{1}$$

Acute toxicities. The acute oral, subcutaneous and intravenous toxicities were determined in male mice (18–22 g), 10 mice being used at each dose level. The LD_{50} values (g/kg) for the various routes were respectively > 5·0, 3·0 and 1·2 g/kg.

Prolonged tests. (1) *Rats.* *BRL.*1621 was administered orally and subcutaneously to groups of 12 male rats over a period of 12 weeks at doses of 500 and 100 mg/kg. A control group of rats received 0·2 ml./100 g normal saline subcutaneously.

During the test, daily weights and food intake were recorded. Weekly observations were made of packed cell volume, white cell counts, and qualitative tests for proteins, sugars and occult blood were carried out on urine specimens. Spectroscopic examination of the blood of the rats in all groups and hæmoglobin concentration was determined on the last week of the experiment.

All rats were killed at the end of the test and post-mortem examinations made. Weights of the spleens, livers, kidneys, adrenals and testes were recorded. In addition to determination of organ weights at autopsy, the following tissues were removed for histological examination: salivary gland, lymph node (mesenteric), thymus, thyroid, heart, lung, liver, kidney, adrenal, spleen, testes, bone marrow, stomach, duodenum, pancreas and small intestine. The tissues at the site of injection were also removed. No hæmatological, biochemical, histological or organ weight abnormalities were observed. Macroscopically the only untoward effect was small areas of necrosis noted at the site of injection in the rats receiving *BRL.*1621 500 mg/kg subcutaneously. These tissue reactions were only seen during the first 3 weeks of the test after which time they disappeared.

(2) *Dogs.* *BRL.*1621 was administered in doses of 2,000 and 500 mg/kg twice daily to groups of 3 dogs for a period of 4 weeks. The following biochemical and hæmatological estimations were made at the beginning and end of the test: ESR (mm/h), PCV (per cent), Hb G (per cent), reticulocytes (per cent), MCHC (per cent), total white cell count and differential count, blood urea (mg per cent), blood glucose

Table 1. PERCENTAGE PROTECTION BY *BRL.*1621, METHICILLIN AND PENICILLIN *G* IN MICE INFECTED WITH *Staph. pyogenes* RUSSELL (PENICILLIN-RESISTANT)

Penicillin	Dose (mg/kg)	Per cent protection	
		Oral	Subcutaneous
*BRL.*1621	200	98·3	97·6
*BRL.*1621	50	58·6	83·9
Methicillin	200	Inactive	84·7
Methicillin	50	Inactive	40·2
Penicillin *G*	200	Inactive	Inactive
Penicillin *G*	50	Inactive	Inactive

(mg per cent) serum proteins—total, albumin, α_1-, α_2-, β- and γ-globulins, alkaline phosphatase KA units, SGPT units and presence of bilirubin. At autopsy the following organs were weighed: brain, heart, lungs, liver, spleen, pancreas, kidneys, thyroids, adrenals and gonads. In addition to these, the histology included examination of: stomach, ileum and colon, cervical and mesenteric lymph glands, salivary glands, skeletal muscle and bone marrow. No hæmatological, biochemical or histological abnormalities were noted.

Local toxic effects. There were no observable tissue reactions to intradermal and intramuscular injection of 1 per cent solutions of the penicillins in groups (3 per group) of guinea pigs and rats 3 days after the injection. The only observable reactions to administration of 10 per cent solutions were a hardening of the skin and erythema around the sites of the intradermal injections.

Following the subconjunctival injection in rabbits of 0·5 ml. of a 50 per cent solution, *BRL.*1621 penetrates into the aqueous humor but an opacity of the cornea develops which persists for at least 14 days with only a slight decrease in the intensity. It would appear, therefore, that *BRL.*1621 is contraindicated for ophthalmological use.

General pharmacology. The effect of *BRL.*1621

Table 2. Minimum Inhibitory Concentration of *BRL.*1621 (Serial dilutions in agar, incubated at 37°C. for 24 hours)

	M.I.C. (μg/ml.)	
	Penicillin *G*	*BRL.*1621
Staph. aureus (6 strains)	0·02–0·05	0·1–0·25
Strep. β-haemolytic (5 strains)	0·002–0·005	0·05–0·1
Strep. viridans (4 strains)	0·002–0·05	0·1
Strep. pneumoniae (5 strains)	0·01–0·02	0·1–0·5
Sarcina lutea	0·005	0·25
Strep. faecalis	12·5	25
E. coli	50	> 250
Proteus vulgaris	50	> 250
Salm. typhi	2·5	> 250
Salm. typhimurium	1·25	> 250
Ps. pyocyanea	> 250	> 250
Shigella shigae	50	50
Aerobacter aerogenes	250	> 250

Table 3. Activity of *BRL.*1621 against fresh Clinical Isolates of Penicillin-resistant Staphylococci compared with that of Methicillin

	M.I.C. (μg/ml.)		
	Penicillin *G*	Methicillin	*BRL.*1621
Staph. aureus strain *C*440	500	2·5	0·5
Staph. aureus strain *C*465	500	2·5	0·5
Staph. aureus strain *C*417	250	2·5	0·5
Staph. aureus strain *C*444	250	1·25	0·5
Staph. aureus strain *C*452	250	2·5	0·5
Staph. aureus strain *C*443	250	1·25	0·25
Staph. aureus strain *C*442	125	2·5	0·5
Staph. aureus strain *C*427	25	1·25	0·25
Staph. aureus strain *C*433	25	1·25	0·25
Staph. aureus strain *C*420	5·0	1·25	0·25
Staph. aureus strain *C*421	2·5	1·25	0·25
Staph. aureus strain *C*438	0·1	2·5	0·5

Table 4. ACTIVITY OF *BRL*.1621 COMPARED WITH *BRL*.1400 USING STAPHYLOCOCCI RECENTLY ISOLATED CLINICALLY

	M.I.C. μg/ml												
	125 or greater	50	25	12·5	5·0	2·5	1·25	0·5	0·25	0·12	0·05	0·02	0·01 or less
Penicillin-sensitive strains (total 152)													
Penicillin *G*											19	63	70
BRL.1400								3	28	74	41	6	
BRL.1621							1		31	81	35		2
Penicillin-resistant strains (total 230)													
Penicillin *G*	81	20	31	49	14	14	3		2	16			
BRL.1400							36	74	87	20	12	1	
BRL.1621							2	66	116	40	5		1

on the blood pressure and respiration was determined in the anæsthetized cat.

Four cats were anæsthetized with a chloralose (1 per cent) urethane (5 per cent) mixture. Doses of up to 100 mg/kg administered intravenously had no effect on the blood pressure but doses in excess of this up to 400 mg/kg had slight hypotensive effects which usually did not exceed 10 mm mercury.

The responses to adrenaline (5·0 μg), acetylcholine (0·2–0·5 μg), histamine (0·5–5·0 μg), were unaffected by doses of 200 mg/kg of *BRL*.1621. Repeated doses of *BRL*.1621 at 5-min intervals increasing from 25, 50, 100, 200 and 400 mg/kg were given. Death occurred when the cat had received a total of 1·6 g/kg.

BRL.1621 penetrates poorly into the C.S.F. After a dose of 500 mg/kg intramuscularly in rabbits the concentration in the C.S.F. was no greater than 2·3 μg/ml. 1 h after administration, while the corresponding serum concentration was 370 μg/ml.

BRL.1621 is eliminated from the body by excretion in high concentration both in the bile and urine of rats. After the oral administration of 100 mg/kg 18·7 per cent is recovered in the urine after 24 h, while after intramuscular administration 42·1 per cent is recovered. 10·2 per cent of the orally administered antibiotic can be accounted for in the bile.

Metabolism in animals. Paper chromatographic examination of the urine collected over 24 h from rats which had received 100 mg/kg *BRL*.1621 intramuscularly, indicated the presence, in small amounts, of two metabolites. Further investigation of these substances is in progress.

Microbiology

Antibacterial activity. Minimum inhibitory concentrations (M.I.C.) for bacteria were determined as described previously[7]. It will be seen from Table 2 that *BRL*.1621 is highly active against *Staph. aureus*, *Strep. pyogenes*, *Strep. viridans* and *Strep. pneumoniae*, but against *Strep. faecalis* and the Gram-negative bacilli the level of activity is very low. Penicillin-sensitive staphylococci were usually inhibited by 0·1–0·25 μg/ml. *Strep. pneumoniae*, *Strep. viridans* and β-*haemolytic Streptococci* were usually inhibited by 0·1 μg/ml.

Penicillin *BRL*.1621 is primarily of interest for its activity against penicillin-resistant staphylococci. Results in Table 3 show that *BRL*.1621 is appreciably more active than methicillin in this respect. Results for the activity of *BRL*.1621 against a larger number of hospital strains of *Staph. aureus* are shown in Table 4. The total of 382 cultures tested

Table 5. ACTIVITY OF *BRL*.1621 COMPARED WITH *BRL*.1400 IN TESTS WITH AND WITHOUT HUMAN SERUM, USING 36 STRAINS OF STAPHYLOCOCCI RECENTLY ISOLATED CLINICALLY

	Serial dilution in agar M.I.C. μg/ml. and number of strains							
	5·0 and over	2·5	1·25	0·5	0·25	0·12	0·05	0·02 and below
Penicillin *G*	20		7		2		3	4
BRL.1621				8	21	6	1	
BRL.1621 +			23	10	3			
BRL.1400				13	17	3		
BRL.1400 +		3	28	5				

+ denotes agar containing 40 per cent human serum

comprised 152 penicillin-sensitive (penicillin G M.I.C. < 0·1 μg/ml.) and 230 penicillin-resistant (penicillin G M.I.C. 0·1 μg/ml. or greater). Taking the penicillin-sensitive strains, only one required a concentration of *BRL*.1621 as high as 1·25 μg/ml. to inhibit growth and approximately 95 per cent of the strains showed M.I.C. values in the range 0·05–0·25 μg/ml. Of the total of 230 penicillin-resistant strains only two required a concentration as high as 1·25 μg/ml. for inhibition and 97 per cent of the strains showed M.I.C. values in the range 0·125–0·5 μg/ml. The largest single group of strains were those with an M.I.C. value of 0·25 μg/ml.

Comparative results are also included in Table 4 for *BRL*.1400 (oxacillin). It will be seen that *BRL*.1621 is slightly more active than *BRL*.1400. Of the total of 230 penicillin-resistant strains, only two showed an M.I.C. as high as 1·25 μg/ml. with *BRL*.1621, whereas with *BRL*.1400 the corresponding number of strains was 36 (15 per cent).

Determination of M.I.C. values in solid media and in broth give similar results and M.I.C. values are not significantly influenced by inoculum size.

Effect of serum on activity. Activity of *BRL*.1621 is depressed by the presence of human serum to an extent somewhat greater than is the case with methicillin. Results are shown in Table 5 for 36 strains of staphylococci. In the absence of serum all were inhibited by 0·5 μg/ml. *BRL*.1621 or less and most of the strains were inhibited by 0·25 μg./ml. In the presence of human serum, however, the majority of the strains required 1·25 μg/ml. to prevent growth. It will be seen from Table 5 that *BRL*.1400 is also influenced by serum, the extent being similar to that with *BRL*.1621.

Bactericidal activity. Viable counts in broth cultures containing *BRL*.1621 were carried out in a manner similar to that of Miles, Misra and Irwin[8]. Penicillin *BRL*.1621 is highly bactericidal to penicillin-resistant staphylococci at concentrations only slightly higher than the M.I.C. values. In bactericidal tests

Table 6. STABILITY OF *BRL*.1621 TO STAPHYLOCOCCAL PENICILLINASE COMPARED WITH *BRL*.1400 AND METHICILLIN

(1) At a concentration of 50 μg/ml. :

Time (min)	μg/ml. remaining			
	Penicillin *G*	Methicillin	*BRL*.1621	*BRL*.1400
0	50 (50)*	50	50	50
15	0 (35)	49	46	43
30	0 (17)	47	42	39
45	0 (6)	46	39	33
60	0 (0)	45	35	27
90	0 (0)	42	28	18

* Penicillinase at a dilution of 1/1,000.

(2) At a concentration of 2,000 μg/ml. :

Time (min)	μg/ml. remaining			
	Penicillin *G*	Methicillin	*BRL*.1621	*BRL*.1400
0	2,000 (2,000)*	2,000	2,000	2,000
15	0 (1,600)	1,840	1,550	1,340
30	0 (1,400)	1,720	1,350	830
60	0 (820)	1,520	800	250

* Penicillinase at a dilution of 1/200

BRL.1621 is substantially more active than methicillin and also slightly more active than *BRL*.1400. At a concentration of 2·0 μg/ml. *BRL*.1621, a kill of approximately 99·9 per cent is obtained in 7 h in broth cultures of penicillin-resistant staphylococci.

Activity of BRL.1621 *by the paper disk method.* Whatman antibiotic assay disks 6 mm diameter were used with two levels of *BRL*.1621, 2 μg and 5 μg per disk. Nutrient agar plates were inoculated with the test organism to result in dense but non-confluent growth and the dishes were left at room temperature for 3–4 h with the disks in place before incubating at 37° C overnight. For a range of staphylococci of varying penicillin *G* sensitivity the zone diameters with *BRL*.1621 showed a fairly good correlation with the M.I.C. values for the strains. Using a 2 μg disk the zone diameters ranged from 19–29 mm, depending on the strain, and with a 5 μg disk the range was 22–33 mm diameter, including the diameter of the disk. Very heavy inoculum, and particularly the presence of blood in the agar, results in smaller zone diameters.

Stability to penicillinase. A penicillinase-producing strain of *Staph. aureus* was grown as described previously[7], and the whole culture, including the cells, was used as enzyme. The tests on penicillinase stability of *BRL*.1621, *BRL*.1400 and methicillin were carried out at two levels of substrate, 50 μg/ml. and 2,000 μg/ml. One volume of the enzyme was added to two volumes of phosphate buffer, *p*H 7, containing the penicillin. The reaction mixture was incubated at 37° C and assays carried out at intervals of time. Because of the extreme instability of penicillin *G* to penicillinase, a parallel test was carried out with penicillin *G* in which the penicillinase was present at a given dilution since with the penicillinase at the concentration used for the methicillin and

isoxazole tests the penicillin *G* was destroyed almost instantaneously. It will be seen from Table 6 that *BRL*.1621 is extremely stable to penicillinase, although slightly less stable than methicillin, and that *BRL*.1621 is slightly more stable than *BRL*.1400. It should be noted that the penicillinase stability of *BRL*.1621, and also that of *BRL*.1400 and methicillin, is greatly influenced by the concentration of the compound present in the test. With these compounds, the concentration of substrate is a limiting factor for penicillinase activity except at very high concentrations and the stability of these compounds is largely due to this effect of substrate concentration on rate of enzyme action. Consequently, the lower the concentration of these penicillins the more stable they become to penicillinase.

Penicillin *BRL*.1621, like methicillin, is a powerful inducer of penicillinase formation.

Emergence of resistance to BRL.1621. By repeated sub-culture of staphylococci in the presence of *BRL*.1621, resistant strains can be selected out in a step-wise manner similar to that seen with penicillin *G*, methicillin and other penicillins. Single step mutants of high resistance to *BRL*.1621 have not been encountered in laboratory experiments with staphylococci. On the other hand, naturally occurring staphylococci resistant to methicillin[9] are also resistant to *BRL*.1621, although since *BRL*.1621 is a more active compound the M.I.C. values with *BRL*.1621 against these resistant strains are lower than those for methicillin. This means that although the M.I.C. values for methicillin may be above the serum levels normally obtained, the same may not be the case with *BRL*.1621. For example, certain strains showing M.I.C. values of 12·5–25 μg/ml. with methicillin were found to be still sensitive to 1·25 μg/ml. *BRL*.1621. In general, the highly resistant strains which showed M.I.C. values of 125 μg/ml. and over with methicillin, also showed M.I.C. values with *BRL*.1621 above the concentrations obtainable in the body but one strain showing an M.I.C. value of 50 μg/ml. with methicillin was still sensitive to 1·25 μg/ml. *BRL*.1621.

Metabolite of BRL.1621. After administration of *BRL*.1621, either orally or intramuscularly, in normal human subjects a small proportion of the compound undergoes a metabolic change in the body to give rise to another penicillin. This metabolite is present in the serum as well as in the urine, and in human beings approximately 10 per cent of the penicillin excreted is in the form of the metabolite. A similar metabolite also occurs with *BRL*.1400.

The metabolite of *BRL*.1621 has been isolated and

found to have antibacterial activity very similar to *BRL*.1621 itself. Moreover, using *Sarcina lutea* and also a penicillin-resistant staphylococcus as test organism, *BRL*.1621 and the metabolite both have the same ring-diameter concentration relationship in the cup-plate assays.

In view of the very close similarity in the biological properties of the metabolite and *BRL*.1621 itself, and also in view of the small amount of the metabolite formed it seems quite certain that the latter does not in any way complicate the determinations of serum levels or the general evaluation of *BRL*.1621.

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FURTHER PHARMACOLOGY AND CHEMOTHERAPY OF CLOXACILLIN

BY

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In concentrations greatly in excess of therapeutic blood levels cloxacillin has a slight hypotensive action. As with other antibiotics, it sometimes causes diarrhoea in rabbits but, in doses up to 200 mg/kg, it does not have a teratogenic effect on the rabbit foetus. Cloxacillin is distributed throughout the body. High concentrations are found only in the liver and kidney, these reflecting the high concentrations in the bile and urine respectively. It differs from other penicillins investigated in that there appears to be little or no renal tubular secretion as demonstrated in experiments on the hen. Cloxacillin is excreted as the unchanged drug and as an active metabolite in the urine and bile. After oral administration it is metabolized in the caecum giving a penicillin which differs from the urinary metabolite and which has a greater antibiotic action against *Sarcina lutea* than the parent penicillin. The activity of the caecal metabolite against resistant and sensitive strains of *Staphylococcus*, however, remains similar to that of cloxacillin. Against infections due to resistant and sensitive *Staphylococcus* in animals cloxacillin is active by both the oral and subcutaneous routes and it is more effective orally than an equal subcutaneous dose of methicillin.

Nayler, Long, Brown, Acred, Rolinson, Batchelor, Stevens & Sutherland (1962) have recently reported on the chemistry, toxicology, microbiology and pharmacology of a new semi-synthetic penicillin, cloxacillin. This penicillin is stable to staphylococcal penicillinase and is active orally. Further studies on the chemotherapy and pharmacology are described in this paper.

METHODS

In all the experimental work cloxacillin was used as its pure sodium salt monohydrate, which is freely soluble in cold water giving a solution stable at 5° C for 1 week. Methicillin (Celbenin) and penicillin G (Solupen) were also administered as their pure sodium salts.

The antibiotics were assayed by the cup-plate technique using *Sarcina lutea* as the test organism. The zone diameters obtained for the control dilution of the antibiotics were plotted against the log of the concentration, and from the regression lines obtained the concentrations of the antibiotics in the specimens were estimated by interpolation. The appropriate dilutions of the controls and samples were made in 0.05 M-phosphate buffer at *p*H 7.0, except that in the experiments where serum concentrations were determined the controls were prepared in serum. Before assay all specimens were stored at 4° C.

Chromatographic studies were carried out using 1 cm wide strips of Whatman No. 1 filter paper 53 cm long with the origin at 11 cm. The solvent was a saturated solution of ether in water buffered to *p*H 6.2 (Karnovsky & Johnson, 1949). 100 ml. of solvent were used

in the troughs of each tank. All tanks were lined with Whatman No. 3 filter paper. The chromatograms were run from 16 to 20 hr at 20° C after equilibration for at least 1 hr.

Detection of the penicillins on the chromatograms was carried out by placing the strips on agar plates seeded with *Sarcina lutea* and incubating overnight at 30° C. The location of the penicillins was seen as clear zones of inhibition of growth on the plates. Control strips were set up using an aqueous solution of cloxacillin.

Cardiovascular and respiratory effects

The carotid arterial blood pressure of cats anaesthetized with a 1% solution of chloralose was recorded with a mercury manometer on a smoked drum. Respiration was recorded by a lever connected by means of a thread sewn to the skin over the xiphisternum. Compounds in 0.9% saline were administered intravenously through a cannula in a femoral vein. The actions of cloxacillin on the blood pressure responses to acetylcholine (4 μg), adrenaline (10 μg) and histamine (2 and 0.5 μg) were observed. The effect on the blood pressure response to stimulation of the uncut cervical vagus nerve (a rectangular-wave stimulus of 2 V and 5 msec duration, at a rate of 20 shocks/sec for 5 sec every 5 min) was also observed.

The electrocardiogram was recorded by an Ediswan pen recorder linked to hypodermic needle electrodes, placed subcutaneously 2 cm on either side of the midline of the anterior thorax. After the administration of cloxacillin, tracings for 30 sec periods were obtained at 2 min intervals. Simultaneous records of carotid arterial blood pressure were obtained by means of a capacitance manometer, and respiratory movements by a thermistor device (Bainbridge, Warren & Wiggins, 1962).

The action of cloxacillin on the superior cervical ganglion of cats was studied by applying rectangular-wave stimuli of 5 msec duration at a rate of 20 shocks/sec for 30 sec every 10 min to the preganglionic trunk. The voltage was adjusted to give just maximal contractions of the nictitating membrane. A similar stimulus was applied to the postganglionic trunk 2 min after the preganglionic stimulation. Responses were recorded by a frontal writing lever on a smoked drum. The effect of intravenous injection of adrenaline (10 μg) on the nictitating membrane was also observed.

Blood concentrations of cloxacillin were determined in each of six cats which had had successive doses of cloxacillin, samples being taken from the left femoral vein every 10 min after the administration of the penicillin.

Pregnancy tests

Six female rabbits of mixed breed and six New Zealand white females of proved fertility were mated. From the 8th day of pregnancy nine of the rabbits (three mixed breed and six New Zealand) were injected intramuscularly with 250 mg/kg of cloxacillin in aqueous solution, and three (mixed breed) were given 180 mg/kg of thalidomide orally. Treatment continued until the 16th day of pregnancy. The animals were observed daily for abortions during the whole course of pregnancy. The animals which came to term had their litters normally.

Absorption of penicillins

Rabbits. 100 mg/kg of cloxacillin, methicillin and penicillin G were administered intramuscularly to groups of five rabbits. Blood samples were removed from the lateral ear vein at 0.5, 1, 2, 4 and 6 hr after administration. The samples were allowed to clot at room temperature and the serum removed for assay.

Dogs. Cloxacillin was administered orally at a dose of 100 mg/kg. Blood samples (0.3 ml.) for assay were taken at 0.5, 1, 2, 3, 4 and 6 hr after administration, by means of a sterile syringe from the radial vein and placed in heparinized tubes containing 5.0 U of heparin in 0.05 ml. of saline.

Distribution and elimination of cloxacillin

Distribution in tissues. The distribution of cloxacillin throughout the body was determined in groups of five male rats, weighing 100 to 200 g, after oral and intramuscular administration of 100 mg/kg. Groups of rats were killed by exsanguination at 0, 0.5, 2, 4, 6 and 24 hr after administration of the penicillin. The urine and faeces from each group were collected and the amounts recorded. The following organs and tissues from the rats receiving the intramuscular dose were removed and weighed: liver, spleen, kidneys, lungs, small intestine, large intestine, caecum, muscles from the site of injection and carcass.

After oral administration of cloxacillin the stomach also was removed. All specimens were homogenized in a "Waring blendor" and appropriate dilutions were made with phosphate buffer at *p*H 7.0 for assay.

Urinary excretion. The mode of urinary excretion in the hen was examined as described by Acred, Brown, Turner & Wright (1961), by stitching a polyethylene funnel over each ureteral opening to collect urine from each kidney. Cloxacillin was dissolved in 2.0 ml. of 0.9% saline and injected into the muscles of the left leg at a dose of 100 mg/kg. Probenecid, which blocks renal tubular secretion, was administered intravenously at a dose of 100 mg.

Biliary excretion. Groups of male rats (weighing 250 to 350 g) were starved overnight. After they had been anaesthetized with ether laparotomy was performed and the bile duct was cannulated with polyethylene tubing (0.4 mm bore). The tubing was passed through a small aperture in the skin at the back of the neck, and the skin and muscle incisions were sutured. The animals were then given an intramuscular injection of 100 mg/kg of cloxacillin and placed in metabolism cages. The polyethylene tubing was taken through the top of the cage and led into a 10 ml. cylinder for collection of the bile. Throughout the 24 hr period samples of the bile and urine were taken.

Cerebrospinal fluid concentrations. The cerebrospinal fluid concentrations of cloxacillin were determined in rabbits after intramuscular administration of 500 mg/kg. 0.5, 1, 2 and 4 hr after administration of cloxacillin two rabbits were anaesthetized with urethane (1 g/kg, intravenously) and cerebrospinal fluid samples were withdrawn from the cisterna magna by means of a sterile syringe and needle. Blood samples were taken from the lateral ear veins at the time of taking the cerebrospinal fluid samples. The blood samples were allowed to clot at room temperature. The serum and cerebrospinal fluid samples were kept at 4° C until assayed.

Investigation of metabolite

In vivo. 100 mg/kg of cloxacillin was administered orally to groups of five male albino rats (weighing 100 to 200 g). At 2, 4 and 6 hr after administration one group was killed and the alimentary tract was removed. The small intestine, caecum and large intestine were separated and the contents removed by washing out with saline. The different parts of the intestine were weighed and homogenized in the same way as in the distribution studies and the homogenates assayed for penicillin. The contents obtained from each section of the intestine were also assayed by the cup-plate technique using *Sarcina lutea, Staphylococcus pyogenes* Oxford and *Staphylococcus pyogenes* Russell (the Russell *Staphylococcus* being resistant to penicillin G). The results were expressed as a concentration in μg/g of tissue or μg/ml. of content and also expressed as a percentage of the dose administered. The extracts and the washings were chromatographed.

In vitro. Homogenates weighing 1 g from various sections of the alimentary canal and the contents were suspended in 3.5 ml. of Krebs solution. To the suspension 0.5 ml. of a 1 mg/ml. solution of cloxacillin was added to give a final concentration of the penicillin in the suspension of 100 μg/ml. The homogenates were placed in a water-bath heated to 37° C and shaken throughout the period of the test. Samples were removed from each homogenate at 0, 2 and 4 hr after adding the penicillin and assayed for penicillin concentration by the usual cup-plate technique using *Sarcina lutea* as the test organism.

Chemotherapy

Thigh lesion tests

Single infection. The protective effect of cloxacillin was determined using the method of Selbie & O'Grady (1954) as modified by Brown & Acred (1960). Groups of ten mice were infected by injecting into the muscle sheath of the left thigh 0.2 ml. of a 1:3 dilution of an overnight growth of *Staphylococcus pyogenes* Russell, type 80. Cloxacillin and methicillin were administered in doses of 50 and 200 mg/kg orally and subcutaneously daily for 3 days, the first dose being given immediately after the infection. The infected control group received no treatment. A non-infected, no treatment, control group was set up with each experiment. The maximum thigh diameters of each mouse were measured with callipers on the 1st, 2nd and 6th days after infection.

Double infection. A double lesion test was also carried out in which the animals were infected with *Staphylococcus pyogenes* Russell in the left hind-limb, and with *Staphylococcus pyogenes* 2187 (penicillin G-sensitive) in the muscles of the right hind-limb. 0.2 ml. of a 1:3 dilution of an overnight culture of each organism was injected into each mouse. Cloxacillin, methicillin and penicillin G were administered in doses of 100 mg/kg orally and subcutaneously, the dose schedule being the same as for the single lesion test.

Daily thigh enlargement was assessed as the difference in thigh diameters between an infected group of animals and the thigh diameters of the non-infected, no treatment, control group. The results were expressed as the percentage protection calculated in the following manner:

$$\frac{\text{Mean daily thigh enlargement of infected control} - \text{Mean daily thigh enlargement of penicillin group}}{\text{Mean daily thigh enlargement of infected control}} \times 100$$

CD50 tests

The effects of cloxacillin, methicillin and penicillin G were also determined in mice infected with *Staphylococcus pyogenes* Smith (penicillin G-sensitive). The method for determination of the LD50's of the culture and the amount of organism given in the infectivity tests were identical to those described by Acred, Brown, Turner & Wilson (1962). For the antibiotics a 4:1 dose ratio was used, the dose being administered in 0.2 ml. of 0.9% saline immediately after the infection. The mice were observed for 4 days and deaths were recorded daily. The percentage of deaths was plotted against dose on log probit paper and the dose of the compound in mg/kg giving protection to 50% of the mice (CD50) was read off from the graph.

RESULTS

Cardiovascular and respiratory effects

Intravenous doses of 25 to 400 mg/kg of cloxacillin administered to anaesthetized cats lowered the blood pressure. With doses below 200 mg/kg the effects were transient and recovery occurred within 5 to 10 min ; after 200 and 400 mg/kg the hypotension was more prolonged, persisting for more than 10 min. Responses to intravenous administration of adrenaline, acetylcholine and histamine, and to electrical stimulation of the uncut cervical vagus nerve, were not significantly altered by cloxacillin. Doses up to 50 mg/kg of cloxacillin had no significant action on the electrocardiogram, while larger doses extended the PQRS complex by 30 to 50% of the control value. Recovery to normal occurred within 8 min of administration of the drug. No significant effects on respiratory movements were demonstrated.

Increasing doses of cloxacillin in the same animal caused an inhibition of up to 67% of the responses of the nictitating membrane to pre- and postganglionic nerve stimulation. A single dose of 200 mg/kg in a previously untreated animal

caused a 32% reduction of the response, but within 5 min it had returned to normal. The responses of the nictitating membrane to adrenaline were, however, unaffected.

Pregnancy tests

Nine out of twelve rabbits in the test became pregnant, six of which received cloxacillin and three received thalidomide. All the rabbits given cloxacillin delivered normal litters; the litter size for the individual animals being (i) 5, (ii) 14, (iii) 8, (iv) 5, (v) 7, and (vi) 1+. The last rabbit (vi) had a litter but, before inspection could be made, it had partially eaten all the young except one, which appeared normal. Examination of the remaining limbs of the young which had been killed revealed no abnormalities. In rabbits (iv) and (v) there was evidence that two embryos in one, and three embryos in the other, had not been maintained after implantation. Of the rabbits which received thalidomide, one showed signs of abortion on the 20th day; this rabbit was killed and there were five underdeveloped foetuses of which four showed spinal deformities or limb abnormalities; two other foetuses had died and at these sites there was bleeding. This condition had given the external appearance of impending abortion. In the second rabbit given thalidomide there was a complete abortion on the 22nd and 23rd days of pregnancy, and in the third rabbit ten undersized foetuses were delivered normally. Four of these showed deformities of the hind-limbs. The mother was killed and the uterus examined. One dead foetus was found which consisted of a mass of disorganized tissue and blood, enclosed by the amniotic membrane.

Absorption

Blood levels after intramuscular administration of the penicillins into rabbits. The blood levels after the intramuscular administration of 100 mg/kg of penicillin G, methicillin and cloxacillin are given in Table 1.

TABLE 1

SERUM CONCENTRATIONS IN GROUPS OF FIVE RABBITS RECEIVING 100 MG/KG OF CLOXACILLIN, METHICILLIN AND PENICILLIN G INTRAMUSCULARLY

		Serum concentration (μg/ml.) at time after dosing			
Penicillin		1 hr	2 hr	4 hr	6 hr
Cloxacillin		10·0	6·6	5·0	4·5
		23·5	17·0	3·6	3·0
		7·2	3·9	4·1	3·1
		8·8	5·0	2·5	4·1
		11·5	7·4	2·1	2·6
	Mean	12·2	8·0	3·5	3·5
Methicillin		12·5	3·65	0·51	0
		18·0	3·65	0·3	0
		14·0	4·2	0·74	0
		18·0	5·0	0·34	0
		14·0	6·2	1·3	0
	Mean	15·3	4·48	0·64	0
Penicillin G		11·0	5·1	2·65	1·13
		9·0	5·0	1·17	0·5
		16·0	10·5	1·0	0·1
		10·5	4·7	0·48	0
		9·8	6·1	1·15	0·23
	Mean	11·26	6·28	1·29	0·39

The patterns of blood level after methicillin and penicillin G are similar, maximum concentrations of 15.3 and 11.3 μg/ml. respectively being obtained 30 min after administration. After 30 min from administration of cloxacillin a concentration of 12.2 μg/ml. was found, but the levels of cloxacillin were more prolonged than those of methicillin or penicillin G, and significant concentrations (3.5 μg/ml.) were detected 6 hr after administration.

Blood levels after oral administration of cloxacillin into dogs. The mean blood levels after oral administration of 100 mg/kg of cloxacillin into dogs are shown in Table 2. A maximum concentration of 4.4 μg/ml. was found 30 min after administration.

TABLE 2

WHOLE BLOOD CONCENTRATIONS OF CLOXACILLIN (μG/ML.) IN DOGS FOLLOWING ORAL ADMINISTRATION OF 100 MG/KG

The dogs weighed from 10 to 12 kg. *No sample

	Whole blood concentration (μg/ml.) at time (hr) after dosing							
Dog	0·5	1·0	1·5	2·0	2·5	4·0	5·0	6·0
1	5·2	3·1	1·8	2·5	0	0	0	0
2	5·4	6·5	3·0	1·0	0	0	0	0
3	3·0	1·8	2·4	0·8	0	0	0	0
4	6·1	*	1·5	1·4	1·0	0	0	0
5	1·8	2·6	1·7	1·0	0·8	0	0	0
6	4·4	2·6	1·3	1·6	1·0	0·8	0	0
7	4·7	2·8	1·7	1·2	1·0	1·1	0·9	0
Mean	4·4	3·2	1·9	1·3	0·54	0·27	0·1	0

Distribution and elimination

Distribution in tissues. The distribution of cloxacillin after oral and intramuscular administration of 100 mg/kg to groups of five male rats is given in Tables 3 and 4. Following intramuscular and oral administration there was no preferential concentration of the penicillin in any particular organ, except in the liver and kidney, the organs specifically concerned with the excretion of the penicillin from the body. The high concentrations found in the sections of the alimentary canal following oral dosing are a consequence of the route of administration. After 6 hr from intramuscular administration, 42.5% of the antibiotic had been recovered in the urine and during the following 18 hr a further 10.6% was obtained.

Following oral administration the total percentage recovered from all sites increased to a maximum of 124% at 6 hr, falling to 63% at 24 hr. While most of the antibiotic was recovered in the urine following intramuscular administration, only approximately 30 to 50% of the total recovered was found in the urine after oral administration. As the antibiotic moved along the alimentary canal from the stomach to the large intestine the concentration of the antibiotic, instead of decreasing as would be expected due to absorption, dilution and loss, actually increased, until at 6 hr the concentration in the large intestine (3,750 μg/ml.) was greater than that found in the stomach at the beginning of the experiment (2,800 μg/ml.).

Urinary excretion in the hen. The mean percentage of the dose excreted by the left kidney during the 1st hr after administration was only 5.8% greater than that excreted from the right kidney (Table 5). Thereafter there was no difference in the

TABLE 3

DISTRIBUTION OF CLOXACILLIN IN TISSUES OF MALE ALBINO RATS AFTER ORAL ADMINISTRATION OF 100 MG/KG

Seven groups of five rats (weighing from 100 to 200 g) were given orally 100 mg/kg of cloxacillin. One group was killed at the end of each time period. The mean concentrations of cloxacillin are expressed in μg/g wet weight of tissue or in μg/ml. of fluids (columns *a*) and the concentration ratios between the tissues (μg/g wet weight) or fluids (μg/ml.) to serum (μg/ml.) are shown in columns *b*. The urinary excretion is expressed as a percentage of the dose administered

Tissue and fluid	Time after administration: 0 hr		0·5 hr		1 hr		2 hr		4 hr		6 hr	24 hr
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*a*
Liver			30·1	3·7	16·5	3·5	9·6	1·3	8·3	2·7	4·8	0
Spleen			2·7	0·3	0	0	2·6	0·4	0	0	1·8	16·7
Kidneys			2·15	0·27	28·0	5·9	12·6	1·8	7·5	2·5	22·1	7·4
Lungs			8·7	1·1	10·2	2·2	9·4	1·3	5·7	1·9	9·9	0
Stomach	2,800·0	143·5	620·0	76·5	485·0	103·2	245·0	34·5	23·5	7·8	11·3	0
Small intestine			440·0	54·3	810·0	172·3	345·0	48·6	142·0	47·3	39·5	15·3
Caecum, large intestine			5·0	0·62	0	0	850·0	119·7	2,000·0	666·6	3,750·0	710·0
Faeces			2·2	0·27	3·3	0·7	4·3	0·6	756·0	252·0	400·0	1,990·0
Carcass	18·2	0·93	4·8	0·6	3·8	0·80	4·5	0·6	3·6	1·2	3·2	2·0
Serum	19·5	(1·0)	8·1	(1·0)	4·7	(1·0)	7·1	(1·0)	3·0	(1·0)	0	0
Urine			176·0	21·7	50·5	10·7	490·0	69·0	360·0	120·0	445·0	267·5
Total recovered (%)	89		48		59		69		83		124	63
% recovered in urine			0·79		1·54		20·2		12·5		29·1	39·8

TABLE 4

DISTRIBUTION OF CLOXACILLIN IN TISSUES OF MALE ALBINO RATS AFTER INTRAMUSCULAR INJECTION OF 100 MG/KG

Seven groups of five rats (weighing from 100 to 200 g) were injected intramuscularly with 100 mg/kg of cloxacillin. One group was killed at the end of each time period. The mean concentrations of cloxacillin are expressed in μg/g wet weight of tissue or in μg/ml. of fluids (columns *a*) and the concentration ratios between the tissues (μg/g wet weight) or fluids (μg/ml.) to serum (μg/ml.) are shown in columns *b*. The urinary excretion is expressed as a percentage of the dose administered

Tissue and fluid	Time after administration: 0 hr		0·5 hr		1 hr		2 hr		4 hr		6 hr	24 hr
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Liver			29·0	2·1	15·2	2·3	2·4	1·3	0	0	0	0
Spleen			48·5	3·6	4·2	0·6	0	0	0	0	0	1·8
Kidneys			242·5	17·6	76·0	11·7	15·2	8·0	3·9	2·1	1·8	1·8
Lungs			15·0	1·1	10·4	1·6	0	0	0	0	0	0
Small intestine			117·0	8·6	166·0	25·5	59·6	31·4	11·1	6·2	6·5	0
Large intestine, caecum			6·0	0·4	0	0	7·6	4·0	127·0	70·5	75·0	1·8
Faeces							0	0	70·0	38·9	359·0	26·4
Right leg (site of injection)	165·0	97·1	260·0	19·3	127·5	19·6	14·0	7·4	1·9	1·0	4·3	0
Carcass	4·7	0·3	23·3	1·7	18·5	2·8	14·2	7·5	0	0	2·3	0
Urine			1,710·0	126·6	2,400·0	369·2	5,400·0	2,842·0	4,780·0	2,665·5	695·0	425·0
Serum	17·0	(1·0)	13·5	(1·0)	6·5	(1·0)	1·9	(1·0)	1·8	(1·0)	0	0
Total recovered (%)	83·4		39·1		40·5		29·8		17·8		51·3	53·7
% recovered in urine			4·2		20·5		15·8		13·7		42·5	53·1

TABLE 5

URINARY EXCRETION OF CLOXACILLIN IN THE HEN

Values are urinary excretions from the left and right kidneys of five hens each of which received 100 mg of cloxacillin. Cloxacillin was administered into the left leg muscles

	Cumulated % of dose excreted at time (hr) after administration											
	Left kidney						Right kidney					
Hen	1	2	3	4	5	6	1	2	3	4	5	6
A	12·4	20·8	24·6	26·2	29·1	29·4	9·1	15·2	19·2	23·6	24·2	24·2
B	9·2	12·3	15·4	17·7	19·7	21·3	17·0	22·7	25·3	26·8	28·8	29·3
C	25·6	30·9	35·3	37·5	40·9	41·7	25·2	32·0	36·8	39·6	40·9	41·9
D	29·6	34·1	38·0	41·5	43·3	44·4	17·2	27·3	30·2	33·4	35·0	36·1
E	35·8	44·6	48·4	51·5	53·6	55·4	15·1	15·3	18·4	20·0	22·1	23·7
Mean	22·5	28·5	32·3	34·8	37·3	38·4	16·7	22·5	26·0	28·7	30·2	31·0

accumulated percentage excreted from the left and right kidneys. After the intravenous administration of 100 mg of probenecid, which blocks renal tubular secretion, the percentages of the antibiotic excreted by the right and left kidneys were almost identical.

Biliary excretion. The concentration of cloxacillin and the percentage of the antibiotic recovered in the bile at hourly intervals over 6 hr, in groups of eight rats administered 100 mg/kg intramuscularly, are given in Table 6. 23% of the antibiotic was recovered in the bile in 6 hr. From an experiment put up in parallel using twelve rats in pairs with collection of urine, 16% of the antibiotic was recovered.

TABLE 6

THE CONCENTRATION OF CLOXACILLIN AND PERCENTAGE RECOVERED IN BILE FOLLOWING INTRAMUSCULAR ADMINISTRATION OF 100 MG/KG

Values are means for groups of eight rats weighing between 250 and 350 g

	Cloxacillin recovered at time (hr) after dosing					
Cloxacillin	0–1	1–2	2–3	3–4	4–5	5–6
Concentration (μg/ml.)	3,862	2,173	978	437	99	51
Cumulated recovery (%)	10·4	18·1	21·1	22·6	22·8	22·9

Cerebrospinal fluid concentrations. After intramuscular administration of 500 mg/kg of cloxacillin the concentration in the cerebrospinal fluid was considerably less than that found in the serum. The results are shown in Table 7.

TABLE 7

CONCENTRATION OF CLOXACILLIN IN SERUM AND CEREBROSPINAL FLUID OF RABBITS AFTER INTRAMUSCULAR ADMINISTRATION OF 500 MG/KG

Values are means of two rabbits per time period

	Concentration (μg/ml.) at time after dosing		
Fluid	1 hr	2 hr	4 hr
Cerebrospinal fluid	2·3	2·3	3·3
Serum	440	142·5	112·5

Metabolism

In vivo. The concentrations of the antibiotic in the intestinal contents and the intestinal walls are given in Tables 8 and 9. As the antibiotic entered the caecum there was a very considerable increase in amount assayable against *Sarcina lutea*,

TABLE 8

CONCENTRATION OF CLOXACILLIN IN THE TISSUES OF RATS AFTER ORAL ADMINISTRATION OF 100 MG/KG

The concentrations were determined by assaying in parallel against *Sarcina lutea*, *Staphylococcus pyogenes* Russell (penicillin-resistant) and *Staphylococcus pyogenes* Oxford (penicillin-sensitive)

	Concentration (μg/ml.) at time after dosing								
	2 hr			4 hr			6 hr		
Tissue and fluid	*Sarcina*	*Staph.* Oxford	*Staph.* Russell	*Sarcina*	*Staph.* Oxford	*Staph.* Russell	*Sarcina*	*Staph.* Oxford	*Staph.* Russell
Small intestine, walls	88·0	76·0	135·0	18·0	0	0	24·0	0	0
Small intestine, contents	1,600·0	1,400·0	1,950·0	245·0	165·0	225·0	110·0	30·0	74·0
Caecum, walls	65·0	3·6	0	460·0	49·0	78·0	210·0	13·0	26·0
Caecum, contents	1,005·0	160·0	56·0	7,300·0	650·0	545·0	8,400·0	350·0	365·0
Large intestine, walls	0	0	0	26·0	0	0	79·0	6·5	16·0
Large intestine, contents	0	0	0	5,150·0	53·0	44·0	4,150·0	505·0	418·0
Urine	935·0	735·0	1,025·0	1,170·0	380·0	650·0	270·0	160·0	230·0

TABLE 9

TOTAL PERCENTAGE RECOVERY OF CLOXACILLIN FROM ALIMENTARY CANAL AND URINE OF RATS GIVEN 100 MG/KG ORALLY

Assays were as in the experiments summarized in Table 8

	Total recovery (% of dose)		
Time after dosing	*Staph.* Russell	*Staph.* Oxford	*Sarcina*
2 hr	35·03	37·5	43·4
4 hr	17·18	19·5	64·4
6 hr	16·5	12·7	135·43

but when assayed against the two strains of *Staphylococcus* there was a considerable reduction in the relative amount recovered. In relation to the amount of metabolite in the intestinal contents the amount in the walls of the intestine was negligible. When the urine from the same animals was also assayed, the concentrations obtained when assayed against the three organisms were of the same order or size. Chromatograms of the samples showed clearly two zones of inhibition for the samples from the caecum and large intestine, one zone corresponding with the parent compound. The second large zone was near the origin (Fig. 1). The R_F value for this latter zone differed from that of the metabolite isolated from the urine.

In vitro. After incubation of the homogenates of various tissues and intestinal contents with cloxacillin there was a considerable increase in the antibiotic activity in the caecal and large intestine contents (Table 10). The initial concentration was 100 μg/ml. but at 4 hr the concentration in the caecal contents was 360 μg/ml. and in the sample from the large intestine was 300 μg/ml. There was a similar

increase in concentration on incubating cloxacillin with faeces. No increase in concentration was recorded with the caecal and large intestine walls, or with any other section of the alimentary canal.

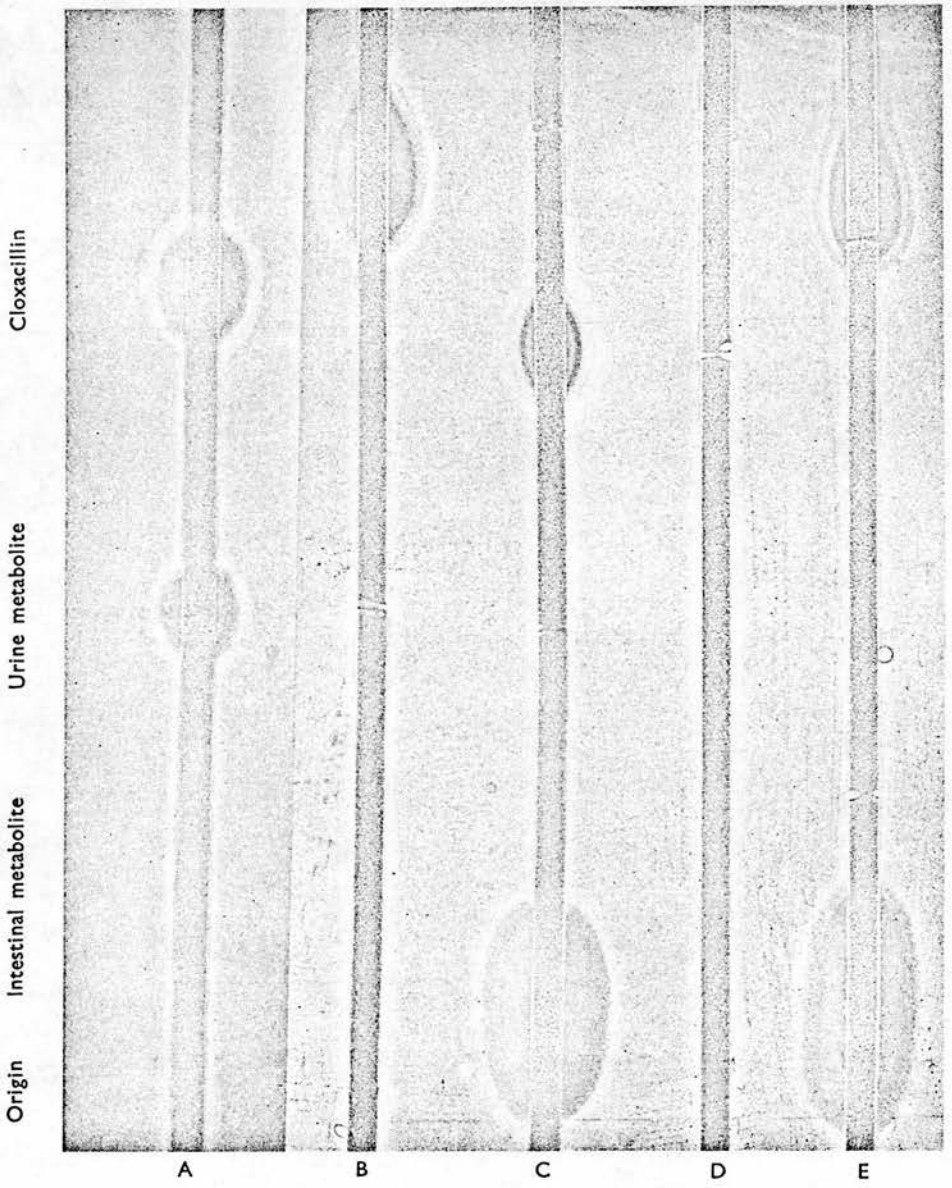

Fig. 1. Chromatography of urine and intestinal homogenates obtained from rats 4 hr after administration of 100 mg/kg of cloxacillin, showing the intestinal metabolite (C and E) and urine metabolite (A). The chromatography paper strips were placed on agar plates seeded with *Sarcina lutea* and incubated at 30° C. A, urine; B, aqueous control; C, caecal contents; D, caecal wall; and E, large intestine contents.

TABLE 10

STABILITY OF CLOXACILLIN IN HOMOGENATES OF RAT TISSUES

1 g of the tissue was suspended in 3.5 ml. of Krebs solution. Cloxacillin was added to give a concentration of 100 μg/ml. and the suspension was incubated at 37° C. At 0, 2 and 4 hr, samples were removed for assay

Tissue and fluid	Concentration (μg/ml.) at time		
	0 hr	2 hr	4 hr
Pancreas	91·0	69·0	66·0
Liver	92·0	62·0	50·0
Small intestine, contents	91·0	71·0	68·0
Small intestine, walls	90·0	73·0	63·0
Duodenum, contents	82·0	43·0	52·0
Duodenum, walls	84·0	86·0	74·0
Control	90·0	91·0	90·0
Caecum, walls	120·0	100·0	70·0
Caecum, contents	110·0	210·0	360·0
Large intestine, walls	115·0	90·0	70·0
Large intestine, contents	105·0	190·0	300·0
Faeces	—	230·0	250·0
Control	96·0	91·0	72·0

Chemotherapy

Thigh lesion test

Single infection. The results of oral and subcutaneous administration of 50 and 200 mg/kg of cloxacillin and of subcutaneous injection of 50 and 200 mg/kg of methicillin on the size of the thigh lesion in mice are given in Table 11. Cloxacillin (200 mg/kg by either route) gave virtually complete healing. Following oral and

TABLE 11

PERCENTAGE PROTECTION AGAINST INFECTIONS IN MICE DUE TO PENICILLINASE-PRODUCING *STAPHYLOCOCCUS PYOGENES* RUSSELL

Doses of 200 and 50 mg/kg of cloxacillin or methicillin were administered to groups of ten mice. Cloxacillin was given orally and subcutaneously, methicillin subcutaneously only

Penicillin	Protection (%)			
	Oral dose		Subcutaneous dose	
	200 mg/kg	50 mg/kg	200 mg/kg	50 mg/kg
Cloxacillin	100·0	60·6	97·7	76·8
	100·0	72·5	100·0	98·1
	100·0	61·7	100·0	93·8
	97·0	61·4	97·5	91·7
	99·0	49·3	99·0	75·9
	100·0	52·0	96·8	84·0
	100·0	54·9	100·0	97·8
Mean	99·4	58·9	98·7	88·3
Methicillin			73·2	24·4
			84·1	31·8
			91·7	61·2
			56·7	43·3
			69·2	38·5
			86·0	36·0
			85·7	57·1
Mean			78·1	41·8

subcutaneous administration of 50 mg/kg, the healing was reduced to 59% and 88% respectively. Administered subcutaneously, 50 mg/kg of cloxacillin gave better protection than 200 mg/kg of methicillin administered by the same route, while 50 mg/kg orally of cloxacillin was superior to 50 mg/kg of methicillin given subcutaneously. Methicillin was inactive orally.

Double infection. The effects of cloxacillin, methicillin and penicillin G, administered subcutaneously and orally to groups of mice, against infections of a penicillin-sensitive *Staphylococcus* in one hind-limb and a resistant *Staphylococcus* in the other hind-limb, are shown in Table 12 and Fig. 2. Cloxacillin was highly effective against both organisms, by both routes. It was not as effective as penicillin G against the sensitive staphylococcal infection, but was considerably more active than penicillin G against the resistant *Staphylococcus*.

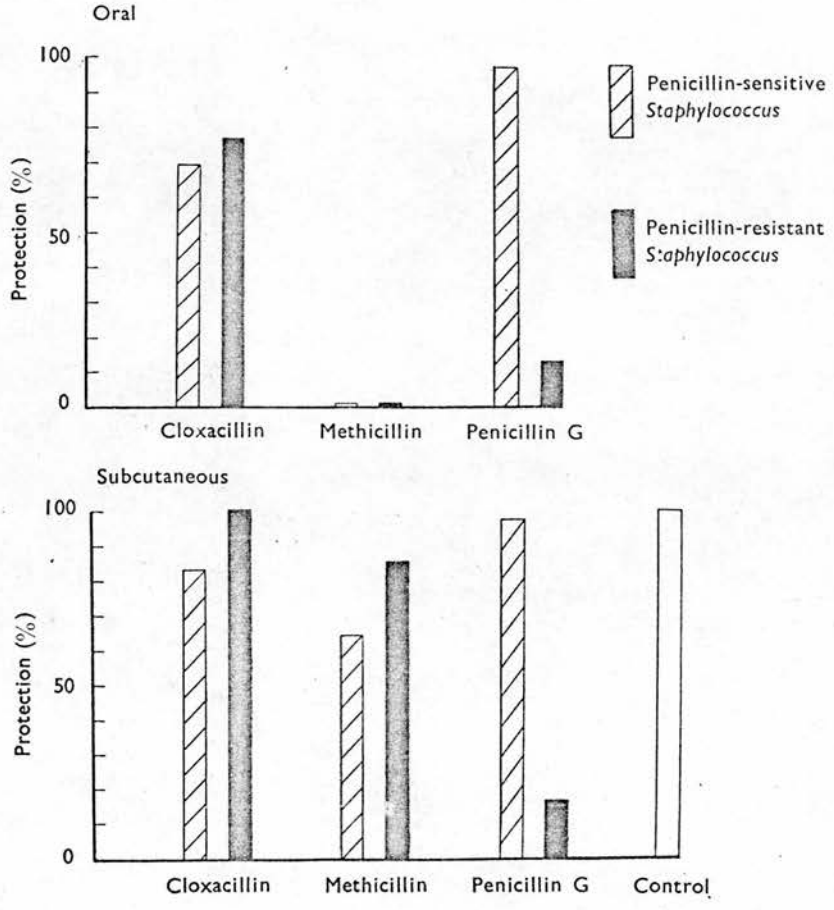

Fig. 2. Histogram showing percentage protection of mice (ten per group) infected intramuscularly with *Staphylococcus pyogenes* Russell (penicillin-resistant) in the left hind-limb and *Staphylococcus pyogenes* 2187 (penicillin-sensitive) in the right hind-limb. 100 mg/kg of cloxacillin, methicillin and penicillin G were administered orally (upper histogram) and subcutaneously (lower histogram) daily for 3 days. The controls were non-infected mice.

TABLE 12

PERCENTAGE PROTECTION AGAINST INFECTIONS IN MICE DUE TO PENICILLINASE-PRODUCING *STAPHYLOCOCCUS PYOGENES* RUSSELL INJECTED INTO THE LEFT THIGH AND PENICILLIN G-SENSITIVE *STAPHYLOCOCCUS PYOGENES* 2187 INJECTED INTO THE RIGHT THIGH

Doses of 100 mg/kg of cloxacillin, methicillin and penicillin G were administered orally and subcutaneously into groups of ten mice. The percentage protection was calculated as described in Methods

	Protection (%) against			
	Staph. pyogenes Russell		*Staph. pyogenes* 2187	
Penicillin	Oral penicillin	Subcutaneous penicillin	Oral penicillin	Subcutaneous penicillin
Cloxacillin	75·6	100·0	69·5	83·1
Methicillin	0	85·2	0	64·4
Penicillin G	13·8	17·3	95·6	98·4

TABLE 13

ACTIVITY OF CLOXACILLIN, METHICILLIN AND PENICILLIN G AGAINST *STAPHYLOCOCCUS PYOGENES* SMITH (PENICILLIN-SENSITIVE)

Activity is expressed in terms of the dose of antibiotic calculated to protect 50% of a group of infected animals (CD50, mg/kg)

	CD50 (mg/kg)	
Penicillin	Oral	Subcutaneous
Cloxacillin	39·0	11·5
Methicillin	Inactive	1·3
Penicillin G	5·8	0·2

CD50 tests

The CD50 values for cloxacillin, methicillin and penicillin G against *Staphylococcus pyogenes* Smith (penicillin G-sensitive) are given in Table 13. Penicillin G was the most effective when given both orally and subcutaneously. Methicillin was only effective by injection, but against this strain of *Staphylococcus* was more effective than cloxacillin.

DISCUSSION

Cloxacillin is one of a series of isoxazol penicillins which is well absorbed orally and is highly effective against staphylococci resistant to penicillin G. After intravenous injection it slightly lowered blood pressure due to a direct action on the heart, and probably to an inhibition of sympathetic nerve conduction. Concentrations in the blood at which these effects were found were in the region of 850 μg/ml. and were therefore very much in excess of those required to produce a therapeutic effect (over 2.5 μg/ml.) and highly unlikely to be encountered clinically.

Cloxacillin, when administered to rabbits, did not induce abnormalities in the foetus, although the mothers may have been affected due to an alteration in the intestinal flora, all the rabbits under test showing varying degrees of diarrhoea. The number of resorption sites which occurred in two of the rabbits was well within the normal expected limits (Adams, 1960), and is of no significance. On the other hand thalidomide killed a number of the foetuses with resulting abortion and in most of the survivors there were signs of limb and other abnormalities.

The effectiveness of cloxacillin against both penicillin-resistant and penicillin-sensitive strains of *Staphylococcus in vivo* has been clearly demonstrated. Cloxacillin when given orally was more active than the same dose of methicillin administered by injection. Against the sensitive *Staphylococcus* cloxacillin was less active than penicillin G. The effectiveness of penicillin G, when administered orally, against the sensitive *Staphylococcus* was probably due to the massive dose of penicillin administered, which would ensure that some of the antibiotic passed through the stomach and was subsequently absorbed giving rise to concentrations in the blood adequate to overcome the infection.

The distribution in the body and the excretion in the bile followed the same pattern as seen with other penicillins (Acred *et al.*, 1961, 1962), and the high concentrations found in the kidney and liver merely reflect the concentrations in the urine and bile. The total amount excreted in the bile was similar to that found with methicillin, ampicillin and penicillin G. The excretion of cloxacillin by the hen's kidney, however, differed in that there was very little renal tubular secretion.

After oral administration of cloxacillin there was an unexpected increase in the amount of antibiotic recovered in the caecum and large intestine, the antibiotic activity in terms of cloxacillin being greater than the amount administered. From these results it was evident that a metabolite was being formed which was more active than cloxacillin against *Sarcina lutea,* but the increased activity was not seen when resistant and sensitive strains of *Staphylococcus* were used in the cup-plate assay. The relative activities of the metabolite of cloxacillin against the two strains of *Staphylococcus* were identical and hence it can be inferred that the metabolite was also stable to staphylococcal penicillinase.

The intestinal metabolite was not absorbed since the conversion occurred in the large intestine and caecum where active absorption is minimal. However, the body can also metabolize cloxacillin producing another active metabolite, which is probably formed in the liver (Mansford, personal communication). This metabolite, which appears in the urine and the bile of rats, is distinct from the intestinal metabolite. Other workers (Vanderhaeghe, Van Dijck, Claesen & De Somer, 1961 ; Rolinson & Batchelor, 1962 ; Rollo, Somers & Burley, 1962) have reported the presence of penicillin metabolites in the urine of humans and it is possible therefore that a number of other penicillins can be metabolized in the body. In estimating penicillins in body tissues, therefore, it is necessary to verify chromatographically as well as by bioassay that the activity measured is due to the administered penicillin and not due to a mixture of the parent penicillin and metabolites. If a mixture is present, spuriously high or low values can be registered since a metabolite can show a different relative activity against the test organism compared to the parent penicillin, as we have found with the intestinal metabolite of cloxacillin when this is assayed against *Sarcina lutea.* Under these circumstances, to obtain values of absolute concentrations it is necessary to isolate the metabolite and determine its *in vitro* activity against the assay organism. In relation to therapeutic activity it is also essential to determine the antibiotic spectrum of the metabolite since it could be more effective against some organisms and less effective against others than the parent penicillin.

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Chemistry and microbiology not carried out under my direction.

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New Semi-synthetic Penicillin active against *Pseudomonas pyocyanea*

Most of the semi-synthetic penicillins developed from 6-amino-penicillanic acid have little or no activity against *Pseudomona pyocyanea*. α-Carboxybenzylpenicillin (carbenicillin : BRL 2064), on the other hand, is active against these organisms and seems to be almost completely non-toxic to man.

by P. ACRED, D. M. BROWN, E. T. KNUDSEN, G. N. ROLINSON and R. SUTHERLAND

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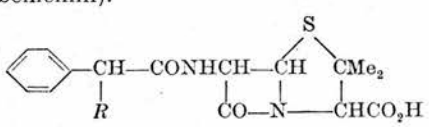
DURING the preparation in these laboratories of many hundreds of semi-synthetic penicillins from 6-amino-penicillanic acid, activity against *Pseudomonas pyocyanea* (*Pseudomonas aeruginosa*) has been found only infrequently. This article describes investigations of the properties and pharmacology of one compound that has been selected for clinical trial. The clinical results (to be reported elsewhere) have shown that carbenicillin has effective anti-*Pseudomonas* activity when given to human subjects by intramuscular or intravenous injection. As with most penicillins, carbenicillin is virtually non-toxic and has no local irritant effects.

Chemistry

The introduction of ionized substituents into the side-chain of benzylpenicillin (I) is known to lead in some cases to enhanced activity against certain Gram-negative bacteria, as in ampicillin (II) which contains a basic α-substituent. We now wish to report on an analogue with an acidic α-substituent, namely α-carboxybenzylpenicillin (III: carbenicillin).



I : R=H (benzylpenicillin)
II : R=NH_2 (ampicillin)
III : R=CO_2H (carbenicillin)

Carbenicillin (which will shortly be available under the registered trademark of 'Pyopen' from Beecham Research Laboratories) is supplied as the disodium salt and contains 5–6 per cent water. It is a white powder which dissolves freely in cold water to give a solution with a *p*H between 6 and 8. Carbenicillin is only moderately stable to acid, the half life at 21° C and *p*H 2·0 being about 140 min and at 37° C and at *p*H 2·0 about 30 min.

On prolonged storage, carbenicillin may decompose to some extent into benzylpenicillin and carbon dioxide. The presence of benzylpenicillin can readily be detected by paper chromatography in butanol–ethanol–water followed by development on an agar plate seeded with *B. subtilis*. Carbenicillin gives a zone of inhibition of bacterial growth near the origin (R_F about 0·03) whereas benzylpenicillin has an R_F of about 0·4. The technique is sensitive because benzylpenicillin is considerably more active than carbenicillin against Gram-positive bacteria.

Microbiology

The antibacterial spectrum of carbenicillin is shown in Table 1. Serial dilutions of the drug were prepared in agar in Petri dishes and the surface of the agar was inoculated using one drop of undiluted overnight broth cultures. The minimum inhibitory concentrations were read after incubation overnight at 37° C. In some respects the antibacterial spectrum of carbenicillin is similar to that of ampicillin. For example, carbenicillin shows significant activity against penicillin-sensitive staphylococci and the streptococci, although this is substantially lower than that of ampicillin or penicillin *G*. Also, carbenicillin is not stable to staphylococcal penicillinase and is relatively inactive against penicillin-resistant strains of staphylococci. Against *Haemophilus influenzae*, *Escherichia coli*, *Salmonella* species, *Shigella* species and *Proteus mirabilis*, carbenicillin shows a similar level of activity to that of ampicillin although, in general, the activity of carbenicillin is somewhat lower. Like ampicillin, carbenicillin shows only a low level of activity against most strains of *Klebsiella aerogenes*. The spectrum of carbenicillin differs from that of ampicillin, however, with regard to *Pseudomonas pyocyanea* and certain *Proteus* species and carbenicillin is primarily interesting because it is active against these organisms.

Activity against Pseudomonas pyocyanea. Compared with the activity one is accustomed to see with most antibiotics of clinical value, the activity of carbenicillin against strains of *Ps. pyocyanea* is low. Not many antibiotics are effective against *Pseudomonas*, however, and in the case of those which are active there are certain problems of toxicity. Carbenicillin, on the other hand, is remarkably free from toxic effects and this allows a dosage to be used which can result in inhibitory con-

Table 1. ANTIBACTERIAL SPECTRUM OF CARBENICILLIN

	Minimum inhibitory concentration (μg/ml.)*
Escherichia coli	5·0
Klebsiella aerogenes	250·0
Salmonella typhi	12·5
Shigella flexneri	5·0
Shigella sonnei	5·0
Pseudomonas pyocyanea	50·0
Proteus mirabilis	2·5
Proteus morganii	5·0
Proteus rettgeri	2·5
Proteus vulgaris	5·0
Haemophilus influenzae	0·5
Staphylococcus aureus Oxford	0·5
Staphylococcus aureus †	50·0
β-Haemolytic streptococcus	0·25
Streptococcus faecalis	25·0
Streptococcus pneumoniae	0·5
Bacillus subtilis	1·25
Sarcina lutea	0·5
Clostridium tetani	0·25
Clostridium welchii	0·25

* Serial dilution in agar; inoculum, one drop of an overnight culture.
† Penicillinase-producing strain.

centrations being reached in the body even though these levels are comparatively high.

The activity of carbenicillin against seventy-four strains of *Ps. pyocyanea* is shown in Table 2. All the strains were recent clinical isolates. It will be seen that a concentration of 50 μg/ml. carbenicillin was required to inhibit the majority of the strains. Some strains were inhibited by 25 μg/ml. but other strains were only inhibited with concentrations as high as 125 μg/ml. Determination of the minimum inhibitory concentrations of carbenicillin is made difficult by the fact that typical strains of *Ps. pyocyanea* do not show a sharp end point when the inoculum is heavy (Table 3). When a heavy inoculum is used, that is, one comprising 10^6 or more cells, confluent growth occurs on agar containing concentrations of carbenicillin up to about 12·5 μg/ml., and at this concentration of drug there is no evidence of any inhibitory effect. At a concentration of 25 μg/ml. carbenicillin, however, growth is extremely scanty compared with the control and the inhibitory effect of the drug at this concentration is very marked, although growth is by no means completely inhibited. At concentrations of carbenicillin above 25 μg/ml., growth is extremely meagre, taking the form of a very thin film which may only just be discernible on the surface of the agar. Consequently, if the criterion of the "minimum inhibitory concentration" (MIC) is one of absolute inhibition of any trace of growth, the MIC with a very heavy inoculum may well be as high

Table 2. ACTIVITY OF CARBENICILLIN AGAINST *Pseudomonas pyocyanea*

	Minimum inhibitory concentration* (μg/ml.) and number of strains					
No. of strains	250	125	50	25	12·5	5·0
74		6	50	15	3	

* Determined by serial dilution of the drug in agar: inoculum one drop of overnight broth culture diluted 1/100.

Table 3. EFFECT OF CARBENICILLIN ON THE GROWTH OF A TYPICAL STRAIN OF *Ps. pyocyanea*

Inoculum	Carbenicillin (μg/ml.) 500	250	125	50	25	12·5	5·0	2·5
Undiluted (about 10^7 cells)	growth barely discernible		sparse growth with some separate colonies			confluent growth		
Diluted 1/100 (about 10^5 cells)	no growth					semi-confluent growth		

Serial dilutions of carbenicillin prepared in agar and the surface inoculated by flooding with overnight broth culture

as 250 μg/ml. or more, as a result of the scanty growth which occurs at concentrations of carbenicillin of 25 μg/ml. and greater. When a smaller inoculum is used in the sensitivity tests, a relatively sharp end point is obtained. With an inoculum of about 10^5 cells a normal, pigmented, semi-confluent growth is obtained on agar with concentrations of carbenicillin up to 12·5 μg/ml., but at 25 μg/ml. and over there is usually no growth at all.

As might be expected, this effect of inoculum size on the MIC of carbenicillin is also seen when the tests are carried out in liquid medium. With a heavy inoculum, growth appears normal with formation of pigment and pellicle up to a concentration of about 12·5 μg/ml. carbenicillin, but at 25 μg/ml. there is no pellicle or pigment and growth is markedly diminished, although complete inhibition of growth may only be obtained with concentrations as high as 250 μg/ml. With a smaller inoculum, a sharp end point is obtained and with most strains of *Pseudomonas* the MIC under these conditions is usually about 50 μg/ml.

This effect of inoculum size on the MIC is outwardly similar to that seen with penicillin *G* and penicillinase-producing staphylococci. Carbenicillin, however, is itself highly stable to the penicillinase produced by typical strains of *Ps. pyocyanea* and the inoculum effect in this case is not due to destruction of drug, nor is the effect due to the growth of resistant mutants present in the original inoculum. When sensitivity tests are carried out on the growth which occurs in the presence of high concentrations of carbenicillin the result obtained is the same as that seen with the original culture, that is, marked suppression of growth at concentrations of 25 μg/ml. and over, but with scanty growth nevertheless persisting up to a concentration as high as 250 μg/ml. when the inoculum is heavy.

Carbenicillin is bactericidal and typical results with a strain of *Pseudomonas* are shown in Fig. 1. For the first 7 h a concentration of 50 μg/ml. usually results in the killing of at least 99 per cent of the original inoculum, but some resumption of growth takes place thereafter which may even increase to a visible amount after 24 h. When this growth is used as inoculum in a repeat test, the same kill is again obtained over the first 7 h followed by a certain amount of growth. The reason for this bactericidal effect

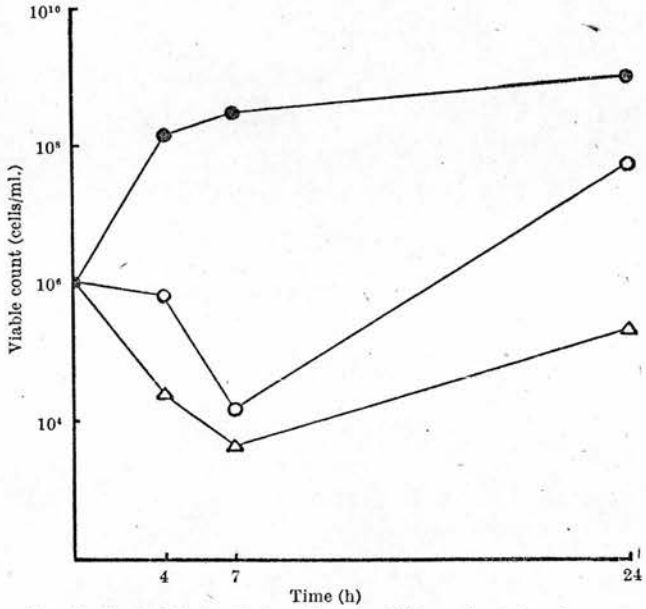


Fig. 1. Bactericidal activity of carbenicillin against *Pseudomonas pyocyanea*. ●, Control; ○, 50 μg/ml.; △, 250 μg/ml.

followed by growth is not known, but it does not seem to be due to the selection of resistant mutants present in the original inoculum.

Activity against E. coli and Proteus species. Table 4 shows the activity of carbenicillin and ampicillin against strains of *E. coli* and *Proteus* species. Against *E. coli*, carbenicillin shows activity equal to that of ampicillin when tests are carried out using dilutions of the drug in agar. An inoculum effect is also seen with *E. coli*, however, similar to that described with *Pseudomonas*. This effect is more marked with some strains of *E. coli* than others and it is also more pronounced when the tests are carried out in liquid medium. Consequently the results of sensitivity tests with *E. coli* carried out in broth frequently show carbenicillin to be markedly less active than ampicillin when the inoculum is large, but comparable with ampicillin in activity when a smaller inoculum is used.

Against *P. mirabilis*, carbenicillin shows the same pattern of activity as does ampicillin, that is, penicillinase-producing strains are resistant while non-penicillinase-producing strains are highly sensitive. Against strains of *P. morganii*, *rettgeri*, and *vulgaris* carbenicillin is interesting in that many such strains which are resistant to ampicillin are relatively sensitive to carbenicillin. This would appear to be due to the greater stability of carbenicillin to the penicillinase produced by these species of *Proteus*.

Table 4. ACTIVITY OF CARBENICILLIN AND AMPICILLIN AGAINST *E. coli* AND *Proteus* SPECIES

Organisms	No. of strains	Compound	Minimum inhibitory concentration (μg/ml.)* and number of strains								
			> 250	250	125	50	25	12·5	5·0	2·5	1·25
E. coli	51	carbenicillin	2					6	18	21	4
		ampicillin	1	1		3		3	35	7	1
P. mirabilis†	6	carbenicillin	5			1					
		ampicillin	5	1							
P. mirabilis‡	14	carbenicillin								3	11
		ampicillin								6	8
P. morganii	5	carbenicillin								4	1
		ampicillin			2	3					
P. rettgeri	5	carbenicillin						3		2	
		ampicillin	3		1	1					
P. vulgaris	6	carbenicillin			1	1		2	2		
		ampicillin	5			1					

* Determined by serial dilution of the drug in agar; inoculum one drop of undiluted overnight broth culture.

† Penicillinase-producing strains.

‡ Non-penicillinase-producing strains.

Effect of serum on activity. Carbenicillin is not highly bound to serum proteins. Sensitivity tests carried out in broth containing 95 per cent human serum show that the activity of carbenicillin is not significantly diminished by the presence of serum, and determinations by an ultrafiltration technique show that 53 per cent of the drug is free in human serum.

Disk sensitivity testing with carbenicillin. Using 6 mm paper disks containing 100 μg carbenicillin, all strains of *Ps. pyocyanea* tested in this laboratory have given zones of inhibitions of 12–20 mm with a heavy inoculum and zones of 18–30 mm with a dilute inoculum. With a heavy inoculum a number of colonies can usually be seen within the zone of inhibition, although when these colonies are picked off and re-tested they show the same sensitivity as the original culture and again give rise to a number of colonies within the clear zone of inhibition.

In the case of Gram-negative bacilli other than *Pseudomonas*, for example, *E. coli* and *Proteus* species, a 25 μg disk would be suitable for sensitivity testing. Using a heavy inoculum, inhibition zones of 20 mm or more are usually obtained with strains which are inhibited by 5 μg/ml. carbenicillin.

Animal Studies

General pharmacology. Carbenicillin in doses up to 500 mg/kg intravenously had no marked effects on the cardiovascular and respiratory systems and the autonomic nervous systems of cats. At 500 mg/kg there was a slight transient hypotensive effect which disappeared within 10–20 min.

Absorption, distribution and metabolism. Carbenicillin is poorly absorbed orally in the rat, but after intramuscular injection the penicillin becomes distributed throughout the body in a similar manner to other penicillins. Relatively high concentrations of the penicillin appear in the alimentary tract after intramuscular administration. Chromatographic examination of intestinal contents and tissue confirms the presence of the drug in the gut. This is probably due to the excretion of bile containing carbenicillin into the intestine.

In order to determine the biliary excretion, the bile ducts of rats were cannulated with 'Polythene' tubing (0·4 mm diameter) and the animals placed in metabolism cages, after being injected with 100 mg/kg carbenicillin intramuscularly. Bile specimens were obtained at intervals of 1 h over a period of up to 6 h after dosing. A total of 15·1 per cent of the penicillin was recovered from the bile during this period and 28·8 per cent of the antibiotic was recovered from the urine over a period of 4 h.

Following intramuscular injection of carbenicillin into rabbits at a dose of 500 mg/kg, samples of cerebrospinal fluid were withdrawn from the cisterna magna at 0·5, 1, 2

and 4 h and assayed. No antibiotic was found in the cerebrospinal fluid, indicating a failure of carbenicillin to penetrate the blood-brain barrier.

When given subconjunctivally, 0·5 ml. of both 10 per cent and 25 per cent solutions of carbenicillin in xylocaine/adrenaline solution B.P. gave concentrations up to 200 μg/ml. in the aqueous humour. Detectable concentrations were present for a longer period after the injection of the 25 per cent solution.

Prolonged toxicity studies in dogs. Thirty pedigree beagle dogs were used in the study. They were housed singly and fed with weighed quantities of dry diet twice daily, the food intake being calculated. Unlimited supplies of water were provided and, in addition, one-third of a pint of milk daily was available.

The animals were divided into five groups of three males and three females and were dosed as follows: group 1, commencing at 2 g/kg/day subcutaneously; group 2, 500 mg/kg/day subcutaneously; group 3, 250 mg/kg/day subcutaneously; group 4, 250 mgzkg/day intravenously; group 5, controls dosed with saline subcutaneously.

Carbenicillin was dissolved in physiological saline so that 1 g of carbenicillin was contained in 1·5 ml. of saline; the solution was made up freshly each day. The dogs were weighed once a week and the dose to be given during the ensuing week was calculated. The intravenous injections were given into the veins of the forelimb. The duration of dosing was 3 months for the subcutaneous groups and 1 month for the intravenous group.

Clinical symptoms were recorded daily, body weight once a week, food intake twice daily and ophthalmoscopic examination was carried out just before the dogs were killed. Haematological, biochemical, urinalysis and serum glucose concentration tests were performed on all dogs, once before dosing began and at 4, 8 and 13 weeks after dosing had begun.

All animals which died during the experimental period were examined after death and all the remaining animals were killed at the end of the dosing period. The internal organs were examined macroscopically and the brain, pituitary, heart, lungs, liver, spleen, pancreas, thymus, prostate/uterus, kidneys, thyroids, adrenals and gonads were removed and weighed. Portions of these tissues, together with sections of aorta, trachea, cervical and mesenteric lymph nodes, salivary glands, various levels of the alimentary tract, gall bladder, skin from injection sites, sciatic nerve, optic nerve, eye, skeletal muscle and bone marrow, were fixed and stained with haematoxylin and eosin. Additional sections of the liver and kidney were specially stained for fat.

There were no deaths from drug toxicity and only one accidental death. There were no observable changes in general physiology, apart from slight local swelling of

tissues around the sites of intravenous injections. Severe local swelling, pain, "cyst formation", but little ulceration, occurred when the subcutaneous route was used for the highest dose level. At 500 mg/kg/day, moderate disturbances resulted, whereas at 250 mg/kg/day there was only minimal evidence of local irritation.

There was some suppression of weight gain associated with a suppression of appetite at the highest dose level by subcutaneous injection, the animals receiving 6–9 g/kg/day. Local irritation rather than the systemic absorption of carbenicillin may have been the chief cause of this loss of weight gain, because while at 250 mg/kg/day by intravenous injection there was no weight suppression, less weight was gained by the same dose level given by the subcutaneous route.

A slight reduction in the serum potassium concentration occurred after 4 weeks at the highest subcutaneous dose level but was not seen later. In two dogs dosed for 4 weeks by intravenous injection at 250 mg/kg, there was some reduction in the concentrating power of the kidney. No effect on the kidney was found in a repeat test at 500 mg/kg intravenously.

Only local tissue damage was found on macroscopic examination at post-mortems and only local irritant reactions at the injection sites were found on histological examination. There was no evidence of organ damage.

Prolonged toxicity studies in rats. Doses of 250, 500 and 1,000 mg/kg carbenicillin were given subcutaneously to groups of thirty Sprague–Dawley caesarian-derived rats from the Charles River Breeding Laboratories, Wilmington, Massachusetts, fifteen males and fifteen females to each group, over a period of 13 weeks. The rats had access to weighed quantities of autoclaved Spillers 'Laboratory Animal' diet and to tap water. The solutions of the drug were prepared freshly each day and administered in a volume of 0·4 ml./100 g. Two injection sites were chosen for each rat, each site being used on alternate days. Throughout the course of the test a record of food consumption and body weight change was kept daily and the mean weekly intake per rat calculated. Urinalysis and haematology were carried out at the beginning of dosing and at 4, 8 and 12 weeks. At the end of the test all the animals were killed by anaesthetization with ether followed by exsanguination.

After the post-mortem examination, all the essential organs were prepared for histological examination. No histological or clinical effects attributable to the drug were detected throughout the course of the dosing apart from a trend in the top group towards a slight fall in the red blood count.

Local irritancy studies. The local action of carbenicillin was determined in the rabbit eye after subconjunctival injection and after topical application. In the first

series of experiments two animals each were allocated to groups which received 0·2 ml. of a 25 per cent, 10 per cent or 1 per cent solution of carbenicillin, respectively. The 25 per cent solution of carbenicillin was prepared in xylocaine/adrenaline solution B.P., in order to reduce the painful response to the hypertonic solution. All the animals were observed twice daily for one week after the injections and any inflammatory responses were recorded. At the end of the period the rabbits were killed and the eyes and surrounding tissues removed for histological examination. The 10 per cent and 1 per cent solutions produced very little reaction with only a very mild inflammatory response, and the histological examination showed no damage. The 25 per cent solution, however, caused inflammation with leucocytic infiltration within 3 h of the injection. Within 48 h, however, the reaction had subsided and by the end of the test period there was no evidence of inflammation.

0·2 ml. of a 25 per cent solution of carbenicillin was applied for 2 min to the eyes of rabbits after which the antibiotic was washed out with warm saline. The eyes of the control animals were treated with saline in the same period of time. The animals were treated twice a day for 3 days. They were then killed on the fourth day and the tissues removed for histological examination. After the first application of the 25 per cent solution there was a slight inflammation of the conjunctiva in one animal which lasted 24 h, but on post-mortem examination no damage was observed; otherwise local application caused no ill effects.

Carbenicillin was also given by intramuscular injection. Twelve rabbits were closely shaven across the dorsum and two injection sites chosen in each of two sacro-spinalis muscles of the back. One site was injected with 1 ml. of a 50 per cent concentration of carbenicillin in saline and the other with 1 ml. of saline. 4 days later the animals were killed and the treated areas prepared for histological examination. At all the sites which received the drug there was fibrolysis and necrosis accompanied in five animals by oedema.

Effects on pregnant rats. Sprague–Dawley caesarian-derived rats from the Charles River Breeding Laboratories Wilmington, were used. The animals were allowed to mate, the day of mating being judged by the appearance of the vaginal plug. Dosing began on day 6 of pregnancy up to and including day 15. Three groups of twenty females were taken, group 1, the control group, being dosed with saline, while groups 2 and 3 received respectively 100 and 500 mg/kg carbenicillin in physiological saline, subcutaneously. The volumes of all injections were standardized to 0·2 ml./100 g body weight. The animals were given free access to Spillers 'Laboratory Animal' diet and to tap water and were closely observed

throughout pregnancy. On day 21 the animals were killed and the uterine contents examined for the number of viable young, resorption sites, litter weight and foetal abnormalities. The young were examined externally after removal from the uterus, one-third being retained for detection of visceral abnormalities and the remaining two-thirds for subsequent dissection of animals followed by clearing and staining of the skeleton by alizarin to detect skeletal abnormalities.

No obvious signs of maternal reactions were observed during the test, but treatment with carbenicillin was associated with a slight retardation in body weight gain. Conception rate, average litter weight and size were not, however, significantly affected by the treatment and there was no incidence of abnormalities due to the drug.

Effect on pregnant mice. Groups of twenty-two mice were mated and treated in a similar way to the rats in the rat pregnancy study except that the animals were killed on the seventeenth day of pregnancy when the uterine contents were similarly examined.

Carbenicillin had no effect on the body weight changes in the mouse, and conception rate, litter size and foetal loss were unaffected at either dosage. There was also no incidence of abnormalities and skeletal deformities due to the drug treatment.

Peri- and post-natal development of the rat. Tests were carried out to determine the effect of carbenicillin on male and female rats before mating and through gestation and lactation to the twenty-first day after birth. Daily doses of 100 and 500 mg/kg of carbenicillin were administered subcutaneously throughout this period.

Parent animals were unaffected, as assessed by daily observation, body weight change, conception rate, length of the gestation period, and parturition. Litter size, litter and mean pup weights and pup loss of rats receiving 500 mg/kg were unaffected at birth or after 4 or 21 days. The increased pup mortality recorded at the lower dosage of 100 mg/kg was therefore considered to be unrelated to the treatment. No abnormal pups were found.

We conclude that carbenicillin at dosages of 500 mg/kg and below does not adversely affect the peri- and post-natal development of the rat.

Absorption and Excretion in Man

The following absorption and excretion studies were undertaken in order to determine a suitable dosage and route of administration for a proposed clinical trial.

Assay methods. The concentration of carbenicillin in serum and in urine was determined by the cup-plate biological assay method. Details of the technique were as described previously[1] except that *Pseudomonas pyocyanea* NCTC 10490 (Ellsworth strain, 1973) was used as the test

organism. *Sarcina lutea* and *Staphylococcus aureus* are unsuitable as assay organisms because carbenicillin contains a small amount of benzylpenicillin and this may appear in the serum and urine together with the carbenicillin. Because *Sarcina lutea* and *Staph. aureus* are many times more sensitive to benzylpenicillin than to carbenicillin the presence of traces of benzylpenicillin may invalidate the assay. *Ps. pyocyanea* NCTC 10490, on the other hand, is sensitive to carbenicillin but is relatively resistant to benzylpenicillin. The culture is unusually sensitive to carbenicillin, showing an MIC of about 1·25 μg/ml., and it is possible to assay concentrations of carbenicillin as low as 2·5 μg/ml. using this strain, while benzylpenicillin at concentrations of up to 500 μg/ml. fails to show any zone of inhibition. Standard solutions of carbenicillin from 1–100 μg/ml. were prepared in human serum, and where necessary the serum samples were diluted with human serum to give a concentration within this range. For the assay of urine samples the standard solutions of carbenicillin were prepared in 0·05 molar phosphate buffer *p*H 7·0 and urine samples were also diluted as required using the same buffer.

Oral administration. After a dose of 500 mg carbenicillin in gelatine capsules to healthy adults in the fasting state, no carbenicillin (that is, < 1·0 μg/ml.) could be detected in serum samples taken at 0·5, 1, 2, 4 and 6 h after administration.

Carbenicillin was detected in the urine, which was collected over the 6 h period after the administration of the drug, but the quantity present in the urine amounted to less than 1 per cent of the dose administered. It would appear, therefore, that carbenicillin is not absorbed to any extent when given by mouth.

Intramuscular administration. Carbenicillin was administered by intramuscular injection to healthy adults in doses of 250 mg, 500 mg and 1 g dissolved in water for injection. The 250 mg dose was dissolved in a volume of 1 ml. and the 500 mg and 1 g doses were administered in a volume of 2 ml. The serum concentrations obtained are shown in Table 5.

It will be seen that intramuscular injection resulted in a high concentration of carbenicillin in serum with a peak at about 1 h after injection. Thereafter the serum levels fell relatively quickly and about 80 per cent of the dose appeared in the urine over the first 6 h.

Table 6 shows results for the effect of probenecid on the serum levels and the urinary excretion of carbenicillin.

Table 5. MEAN SERUM CONCENTRATIONS OF CARBENICILLIN AFTER INTRAMUSCULAR INJECTION IN ADULT VOLUNTEERS

No. of subjects	Dose (mg)	Serum concentration (μg/ml.) 0·5 h	1 h	2 h	3 h	4 h	6 h
10	250	5·9	7·9	5·7	2·6		
9	500	13·7	17·7	13·2	–	2·0	
16	1000	21·6	25·3	22·1	–	10·9	3·7

Table 6. EFFECT OF PROBENECID ON SERUM LEVELS AND URINARY EXCRETION OF CARBENICILLIN IN HEALTHY ADULT VOLUNTEERS

	No. of subjects	Mean serum concentration (μg/ml.) 0·5 h	1 h	2 h	4 h	6 h	Percentage of dose excreted in urine over 0–6 h
With probenecid*	10	25·7	39·9	48·0	27·4	19·4	48
Without probenecid	10	26·8	29·4	22·2	10·8	3·3	84

Carbenicillin dose 1·0 g by intramuscular injection.

* 1 g 1 h and 10 h before administration of carbenicillin.

Probenecid was administered to healthy adults in a dose of 1 g 10 h, and again 1 h, before the intramuscular administration of 1 g carbenicillin. It will be seen that probenecid produced a substantial increase in the peak levels of carbenicillin obtained in serum and the rate of elimination of the drug from the serum was markedly diminished.

Chromatography of human urine, obtained after intramuscular administration of carbenicillin, indicated that the drug does not undergo any substantial metabolism in the body.

Intravenous administration. Results are shown in Table 7 for the serum levels obtained after intravenous injection of 1 g carbenicillin. This amount was dissolved in 10 ml. water and administered over a period of 3 min. Although the drug was eliminated rapidly from the blood it will be seen that serum levels in excess of 100 μg/ml. were obtained during the first 30 min after administration of this dose.

Table 7. SERUM CONCENTRATION OF CARBENICILLIN AFTER INTRAVENOUS ADMINISTRATION OF 1 g

Subject	Serum concentration (μg/ml.) 0·25 h	0·5 h	1 h	2 h	4 h	6 h
A	140	127	60	28	3·7	< 3·0
B	140	114	55	32	4·4	< 3·0

Carbenicillin levels in serum and in urine in relation to antibacterial activity. As a result of the extensive elimination of the drug by way of the kidneys, relatively high concentrations of carbenicillin are obtained in the urine. In the studies involving a dose of 500 mg by intramuscular injection, the concentration in the urine collected over the first 6 h was generally 1,000–2,000 μg/ml. This is considerably in excess of the concentration of drug required to inhibit the growth of *Ps. pyocyanea*. On the other hand, it is questionable whether intramuscular injection of 1 g carbenicillin at intervals of 6 h, even in conjunction with probenecid, would result in tissue concentrations which would be inhibitory towards typical strains of *Ps. pyocyanea*. The minimum inhibitory concentration of carbenicillin for most strains of *Ps. pyocyanea* appears to be 50–100 μg/ml., and although the drug is not highly bound to serum protein (about 50 per cent is bound) it would probably be desirable to achieve serum levels some-

what in excess of 100 μg/ml. This can readily be attained by intravenous administration.

In addition to the activity which carbenicillin shows against *Ps. pyocyanea*, the drug is active against other pathogens, including *E. coli*, and also strains of *Proteus morganii*, *P. rettgeri* and *P. vulgaris* which are generally resistant to ampicillin. On the basis of the *in vitro* sensitivity of these latter organisms to carbenicillin, the serum concentrations achieved by intramuscular administration of the drug might be adequate and the levels attained in urine are again greatly in excess of the minimum inhibitory concentrations.

The results obtained in these studies suggested that for clinical trials on the therapeutic effectiveness of carbenicillin in urinary tract infections, a suitable dosage might be 1·0 g by intramuscular injection at intervals of 6 h. The treatment of pseudomonas septicaemia, wound infections and tissue infection in general would require the intravenous infusion of carbenicillin (with or without probenecid) in order to attain inhibitory serum and tissue concentrations.

We thank our colleagues for experimental assistance and Mr F. P. Doyle for advice and guidance. We also thank Dr A. N. Worden and his colleagues at Huntingdon Research Centre for their assistance with the long-term toxicity studies on carbenicillin (BRL2064).

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[1] Knudsen, E. T., and Rolinson, G. N., *Brit. Med. J.*, ii, 700 (1960).

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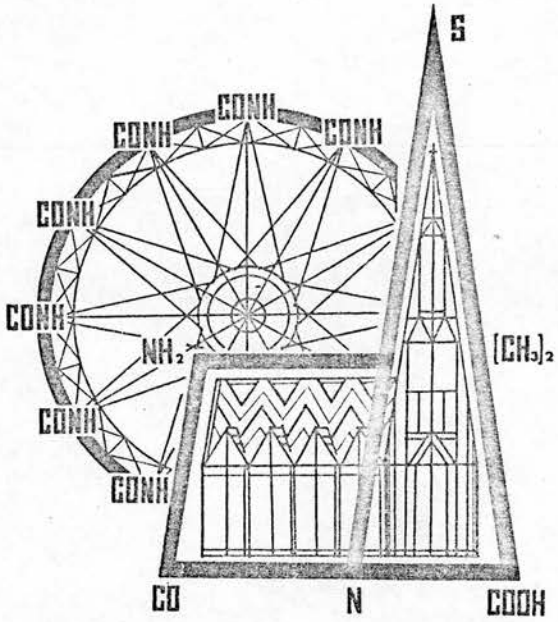

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26. Juni — 1. Juli	June 26 — July 1	26 Juin — 1 Juillet

SEPARATUM

PHARMACOLOGY AND TOXICOLOGY OF A NEW SEMI-SYNTHETIC PENICILLIN BRL 2064 (CARBENICILLIN)* ACTIVE AGAINST PSEUDOMONAS AERUGINOSA

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α-Carboxybenzyl penicillin (BRL 2064) is a new semi-synthetic penicillin which has been shown to have interesting activity against Pseudomonas aeruginosa (Sutherland and Rolinson, 1967). This paper describes some aspects of the toxicology and pharmacology.

TOXICOLOGY

BRL 2064 is virtually non-toxic when administered subcutaneously, intramuscularly and intravenously to rats and mice (Table I).

Species	Route	LD_{50} mg/kg
Mouse	i.m.	> 10,000
	s.c.	> 4,000
	i.v.	> 10,000
Rat	s.c.	> 5,000
	i.v.	> 5,000

Table I
Acute Toxicity of BRL 2064 in Rats and Mice

Prolonged administration studies were carried out in rats and dogs over a period of 13 weeks. Subcutaneous doses of 250, 500 and 1000 mg/kg/day were administered to the rats and 250, 500 and 2000 mg/kg/day to the dogs. The high dose in dogs was increased at three weeks to 3000 mg/kg/day and subsequently reduced because of local reactions. A further group of dogs was dosed intravenously with 250 mg/kg/day for a period of four weeks.

Clinical symptoms were observed daily, body weight once per week, food intake twice daily and opthalmoscopic examination was carried out just prior to termination. Haematology, biochemistry, urinalysis and S.G. concentration tests were carried out before dosing commenced, and 4, 8 and 13 weeks after dosing had begun. Selected organs were weighed

* Pyopen is the registered trade mark of Beecham Research Laboratories for carbenicillin.

and histological examination carried out on samples of most body tissues.

There were no deaths due to drug toxicity during the course of test and no clinical symptoms were seen which were indicative of systemic toxicity. Local reactions of increasing severity with increasing dose were seen in the dogs receiving BRL 2064 subcutaneously. The highest dose group showed localised swelling within two days of dosing beginning. The irritation in this group became increasingly worse and the dose was progressively reduced from the 5th week of the test and stopped at the 9th week. The reactions were less marked in the group receiving 500 mg/kg and there was only slight evidence of irritation from the 9th week onwards in the group receiving the 250 mg/kg dose. Actual ulceration of the skin was rare.

Post mortem examination revealed only local tissue reactions, there being no macroscopic organ changes. Apart from a slight increase in the weights of the kidneys and adrenals obtained from dogs receiving the highest subcutaneous dose, no organ weight changes were observed. Histologically, apart from local irritant effects characterised by tissue necrosis, haemorrhage and marked fibroblastic reaction, there was no evidence in any of the tissues examined of alterations in morphology which could be attributed to the administration of BRL 2064.

Teratogenic studies were carried out in rats and mice using the protocol recommended by the F.D.A. Doses of 500 mg/kg and 100 mg/kg BRL 2064 administered subcutaneously were used in the study. Conception rate, average litter size and weight were not significantly affected and there was no evidence of abnormalities.

Peri- and post-natal development of rats was unaffected by subcutaneous administration of doses of 500 and 100 mg/kg BRL 2064.

PHARMACOLOGY

Distribution and Metabolism in Rats

The distribution of BRL 2064 following intramuscular administration of 100 mg/kg was studied in rats. BRL 2064 was found to be distributed throughout the body in a manner similar to other penicillins. No preferential concentration of the penicillin in any particular tissue was found apart from the organs associated with elimination of the penicillin from the body, i.e. liver and the kidney. Chromatographic examination of intestinal contents and tissue

homogenates revealed unchanged BRL 2064 in the samples, indicating little or no metabolism of the penicillin within the body. However, chromatographic examination of the urine from rats following intramuscular administration revealed a trace of a metabolite which was active against Sarcina lutea but had no activity against Pseudomonas.

Biliary and Urinary Excretion in Rats

Excretion of BRL 2064 in bile was examined in conscious rats (375 - 475 g.) following cannulation of the bile duct. Experiments were also set up in which urine was collected from normal unoperated rats. The penicillin was administered intramuscularly in a dose of 100 mg/kg.

The results obtained with BRL 2064 are shown in the accompanying table and compared with the results we have obtained for other new penicillins and penicillin G.

Penicillin	Mean Concentration µg/ml at hours after dosing			% of dose recovered in bile	% of dose recovered in urine
	0 - 2	2 - 4	4 - 6		
BRL 2064	1984.0	54.0	1.75	15.1	28.8
Cloxacillin	3017.0	707.0	75.0	23.0	16.0
Ampicillin	1835.0	469.0	-	9.6	31.3
Benzyl (G)	1934.0	476.0	183.0	15.0	25.0

Table II

Excretion of Penicillins in Bile and Urine of Conscious Rats following intramuscular administration of 100 mg/kg

BRL 2064 is excreted in the bile and urine in a manner similar to other penicillins. The high concentration found in bile, the ratio of the concentrations in bile compared to that found in blood are in the range 200 - 500, contrasts markedly with those found for other antibiotics. Stewart and Harrison (1961) using a technique similar to that described above found for streptomycin, neomycin, paramomycin and chloramphenicol, that the concentrations in the bile were very much lower than those found in blood. Streptomycin gave the highest yield in the bile, the amount excreted being only 0.08% of the administered dose. These results emphasise a property which distinguishes the penicillin from most other antibiotics, namely

their excretion in high concentrations in the bile.

Penetration into the Cerebrospinal Fluid (C.S.F) of Rabbits

Only trace concentrations of BRL 2064 were found in the C.S.F. of rabbits at intervals up to 4 hours following intramuscular administration of 500 mg/kg of the penicillin. Blood samples removed at the same time as the C.S.F. samples contained high concentrations of the penicillins. Penicillins in general do not penetrate the C.S.F. readily and the results shown in Table III compare those found for BRL 2064 with other penicillins.

Penicillin	Concentration µg/ml at hours after dosing					
	1.0		2.0		3.0	
	C.S.F.	Serum	C.S.F.	Serum	C.S.F.	Serum
BRL 2064	Trace	72.5	Trace	45.0	Trace	8.0
Cloxacillin	2.3	440.0	2.3	142.5	3.3	112.5
Ampicillin	1.4	54.0	0.1	14.2	0.05	4.2
Benzyl (G)	0.15	130.0	0.43	38.0	0.05	200.0

Table III

Concentrations of Penicillins in the C.S.F. and Serum of Rabbits following intramuscular administration of doses of 500 mg/kg

Penetration into the Aqueous Humour of Rabbits

10% and 25% solutions of BRL 2064 were prepared using xylocaine 2.0% + adrenalin injection solution B.P. and 0.5 ml. injected subconjunctivally into groups of rabbits 1.5 - 2.0 kg. At 0.5, 1 and 2 hours after dosing the rabbits were anaesthetised and the aqueous humour withdrawn from the injected eye. Cardiac blood samples were taken at the same times. The results, together with those found for other penicillins, are shown in Table IV.

BRL 2064, as found with cloxacillin and ampicillin, readily penetrates into the aqueous humour. A moderate transient inflammation of the subconjunctival tissue was seen following injection of the 25% solution of BRL 2064. No adverse responses were seen following injection of the 10% solution.

Penicillin	Dose %	Concentration µg/ml at hours after injection					
		0.5		1.0		2.0	
		Aq. humour	Blood	Aq. humour	Blood	Aq. humour	Blood
BRL 2064	10	200.0	10.8	110.5	0	0	0
BRL 2064	25	203.0	24.1	45.5	6.0	7.9	0
Ampicillin	25	60.0	12.3	48.0	5.6	19.0	0.4
Cloxacillin	50	-	-	660.0	98.0	210.0	16.0

Table IV

Penetration of Semi-synthetic Penicillins into the aqueous humour of Rabbits following subconjunctival administration

CONCLUSIONS

BRL 2064 is a new semi-synthetic penicillin active against strains of Pseudomonas, which is distributed throughout the animal body in a manner similar to other penicillins. High concentrations appear in bile and urine, and the penicillin penetrates readily into aqueous humour following subconjunctival administration. As with other penicillins, BRL 2064 does not penetrate readily into the cerebro-spinal fluid. Carbenicillin is non-toxic and can be administered in high doses over long periods of time to dogs and rats. Some local irritation at the sites of subcutaneous injection were seen in animals receiving high doses of the penicillin. No effects on foetal, peri- and post-natal development were observed in mice, and rats, following administration of high doses of BRL 2064.

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SOME CHEMOTHERAPEUTIC AND PHARMACOLOGICAL ASPECTS OF THE NEW SYNTHETIC PENICILLINS

by

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From the Department of Pharmacology and Chemotherapy, Beecham Research Laboratories, Brockham Park, Betchworth, Surrey.

(Presented at the Xth General Meeting of Japan Society of Chemotherapy, on June 8, 1962, in Tokyo, Japan)

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The concept of chemotherapy as advocated by EHRLICH received considerable impetus by FLEMING's discovery of penicillin in 1929. However, the full significance of FLEMING's work was not fully appreciated until ABRAHAM, CHAIN, FLOREY and their colleagues in 1941[1] isolated natural penicillin in sufficient quantities for clinical evaluation.

The remarkable clinical effectiveness of penicillin stimulated a flood of research into the development of new natural antibiotics. Many of these, however, proved to be toxic and their side effects restricted their application. Nevertheless, this work diverted attention from penicillin, and research work in the penicillin field was mainly confined to studying possible modifications produced by the addition of different precursors to fermentation brews.

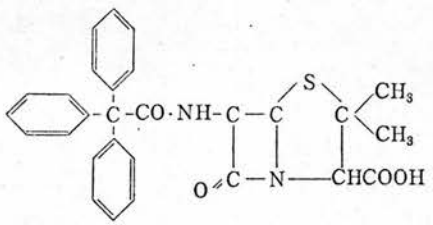
However, the research team at Beecham Research Laboratories in collaboration with Professor CHAIN, commenced an investigational programme with a view to the chemical modification of p-aminobenzyl penicillin. During the course of this work a marked discrepancy between the corresponding biological assays and the chemical assays was found. The cause of the discrepancy was investigated and this resulted in the recognition and subsequent isolation of 6-APA by my colleagues[2] (BATCHELOR, DOYLE, NAYLER and ROLINSON). We were now in a position to prepare numerous new penicillins by semi-synthetic routes which were hitherto unaccessible by the previous fermentation methods. It was hoped that by a systematic study of the new compounds it would provide in the first instance a lead to the development of a new penicillin which would be effective against staphylococci previously insensitive to the natural penicillins and which would still retain the character of non-toxicity. Our second objective was the development of another penicillin which would be effective against a wider range of organisms than penicillin G, particularly in the gram negative field.

In our approach to these studies we went on the assumption that *in vivo* activity might differ from *in vitro* activity. For example, a more active metabolite may be formed in the body or the activity of the antibiotic may be potentiated *in vivo* by some other mechanism. As far as was possible, therefore, all compounds which by *in vitro* tests were more stable to penicillinase than penicillin G were tested in the animal for activity against a penicillin-resistant and penicillin-sensitive staphylococcus.

The activity of the penicillins against penicillin-sensitive *Staphylococcus aureus* (SMITH) was assessed by determining the dose of penicillin needed to cure 50% (CD_{50}) of a group of mice infected intraperitoneally with a large dose of the organism. Activity against infections due to penicillin resistant *Staphylococcus pyogenes* (RUSSELL) was determined by the thigh lesion method described by SELBIE[3] and as adapted by BROWN and ACRED[4] for evaluating antibiotics.

In the first stages of our investigation we prepared a large number of different penicillins. Amongst these was tri-phenylacetyl penicillin :

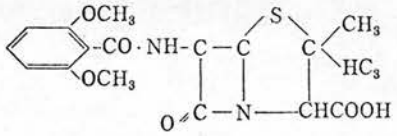
Fig. 1 Tri-phenylacetyl penicillin



This penicillin proved *in vitro* to be stable to Staphylococcal penicillinase and was active against the majority of resistant staphylococci at minimal inhibitory concentrations of 1~2 μg/ml. However, *in vivo* the compound proved to be completely inactive. No blood levels could be obtained as it was highly protein bound and was held firmly to the tissue proteins at the site of injection. In addition, after intravenous administration it proved to be toxic and therefore of no value clinically. This work, however, gave us a major pointer to the production of new penicillins which would be stable to penicillinase. It would appear that the steric configura-

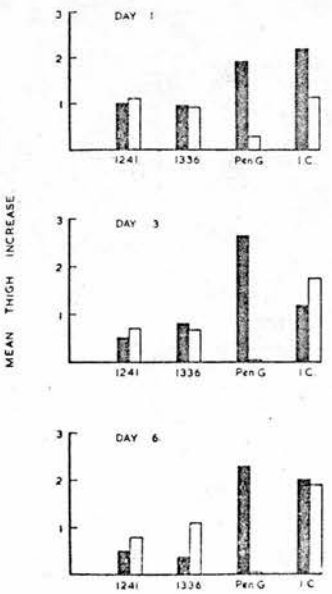
tion of the side chain prevents attachment of the enzyme and therefore prevents the disruption of the nucleus. A number of penicillins were now prepared which by their structural configuration would prevent the access of the enzyme to its points of attachment. This work led very quickly to the synthesis of 2:6-dimethoxybenzyl penicillin which has proved to be highly effective clinically and free from side effects.

Fig. 2 2:6-dimethoxybenzyl penicillin



A large number of analogous compounds to methicillin have also been prepared, but only a few have been shown to possess activity of the same order against both sensitive and resistant staphylococci. Derivatives of 1-naphthoic acid were among the more active of the new compounds. We therefore assessed these compounds by the 'double lesion'. In this procedure we injected the sensitive strain into one hind limb and the resistant strain into the other hind limb of a mouse. Groups of 10 animals were used and these were treated subcutaneously with the antibiotic for 3 days, and the mean increase in the thigh diameter was estimated and compared with the non-treated and non-infected control group.

Fig. 3 Mean increase compared with non-infected controls in thigh diameters of mice (10 per group) infected in the right thighs with *Staphylococcus pyogenes* (Russell)-a penicillin resistant staphylococcus, and infected in the left thighs with *Staphylococcus pyogenes* 2187-a penicillin sensitive staphylococcus. The mice were treated daily for three days with 100 mg/kg subcutaneously with each penicillin. I. C.-infected non-treated control group.

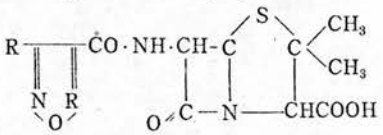


A comparison of 2-methoxynaphthyl penicillin and 2:6-dimethoxybenzyl penicillin is shown in Fig. 3. The effectiveness of both compounds against both the resistant and sensitive strains is demonstrated whereas penicillin G is only effective against the sensitive strain.

The penicillin derivatives of the following acids had little or no activity: Quinoline-4-carboxylic acid, Pyrimidine-5-carboxylic acid, Pyrazole-4-carboxylic acid, Indole-2-carboxylic acid, Benzofuran-2-carboxylic acid, Furan-3-carboxylic acid, Thiophene-2-carboxylic acid and Thiazole-4-carboxylic acid.

All of the preceding penicillins, however, have proved to be inactive orally, but a significant advance has been made recently with the synthesis in our laboratories of a series of isoxazolyl penicillins[5]. The general formula of this series is shown in Fig. 4.

Fig. 4 Isoxazolyl penicillins



R=alkyl or aryl radical

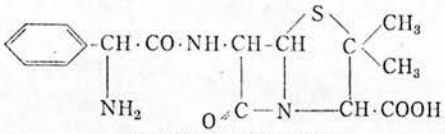
A number of these compounds have proved to be considerably active against both resistant and sensitive staphylococci. Among the more active of the compounds, however, the results *in vivo* show that there is little to choose between any of these compounds by the oral or intramuscular routes. One of these compounds, BRL. 1400 (5-methyl-3-phenyl-4-isoxazolyl penicillin), has been examined extensively by Bristol Laboratories[6] and this compound is now in clinical use. We are continuing our examination of the series and have found that some have given superior blood levels in humans to BRL. 1400 (KNUDSEN, personal communication). Unfortunately detailed toxicity studies and clinical assessment have still to be completed, and it is too early at this stage to elaborate further.

Broad-spectrum Penicillin

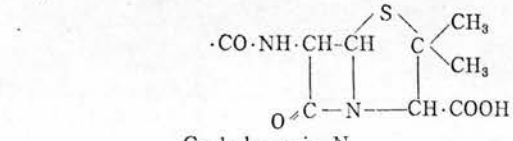
Penicillin G has a slight but significant activity against certain gram negative organisms *in vitro* but in general most of the new synthetic penicillins do not possess activity in this respect. However, we noted that cephalosporin N, which has an amino group in the side chain, showed a slight enhancement in activity. We felt, therefore, that the amino group might play a significant part in promoting activity against gram negative organisms.

Consequently, we prepared a number of penicillins

Fig. 5



Ampicillin-BRL. 1341



Cephalosporin N

with amino acid side chains and of these, α-aminobenzyl penicillin proved to be very active, and from the *in vitro* investigations by ROLINSON and STEVENS[7] it was shown to be slightly but significantly more active than chloramphenicol. We therefore carried out a comparison of the effect of ampicillin with chloramphenicol, tetracycline and streptomycin[8] in mice infected with both gram negative and gram positive organisms. The results *in vivo* clearly confirmed the *in vitro* results.

In these experimental infections, ampicillin was shown to be excedingly effective against both types of organisms. Clinically, particularly against the coliform group of organisms associated with urinary tract infections[9], the experimental expectations have been fully realised although the effects against active typhoid have been somewhat disappointing, but there are indications with increased and more frequent dosing better clinical responses can be obtained. Additional clinical tests show that ampicillin is also very effective in cases of chronic bronchitis, particularly those associated with *haemophilus influenzae*. The particular merit, however, of ampicillin is that it is devoid of systemic toxicity, and apart from subjects who are already sensitive to penicillin it can be administered safely in large doses. Most other oral antibiotics suffer from untoward toxic effects which can limit their usefulness.

General Pharmacology of Methicillin and Ampicillin

With regard to toxicity both drugs are remarkably free from acute toxic effects in the same way as penicillin G. In doses up to 3 g/kg both orally and subcutaneously in rats and mice no toxic symptoms have been seen with methicillin, but in the range of 3～5 g, occasional mild convulsions have occurred[10] (ACRED, BROWN, TURNER and WRIGHT). With ampicillin no toxic effects have been observed with doses of up to 5 g/kg[11] (ACRED, BROWN, TURNER, WILSON). In view of the very low order of toxicity we have not tested larger doses. Ampicillin and methicillin have also no long term toxic effects. In prolonged tests in dogs where the drugs were administered at 500 mg/kg daily for 30 days, neither compound has given rise to any sign of toxicity. Neither has the intramuscular injection of a 10% solution of each antibiotic caused a significant degree of irritation.

Absorption and Distribution

Methicillin is not absorbed orally, but by intramuscular injection it gives good blood levels which are comparable with penicillin G. On the other hand, ampicillin is well absorbed orally and intramuscularly in dogs. If administered orally it in fact gives superior levels to penicillin V, though in humans there is no clear advantage (KNUDSEN, personal communication).

Both antibiotics after intramuscular administration are similarly distributed throughout the body. Apart from the liver and kidney neither antibiotic is concentrated particularly in one organ. The high concentrations of antibiotic in these two organs, however, probably reflect the concentration of the antibiotic in the bile and urine and in no way represents an actual concentration in the tissue

Table 1 Activity of ampicillin, chloramphenicol, tetracycline and streptomycin against gram negative and gram positive infections in mice. The results are expressed in terms of the dose of antibiotic, administered either orally or subcutaneously, calculated to protect 50% of a group of infected mice (CD_{50} mg/kg).

Infecting organism	CD_{50} mg/kg							
	Ampicillin		Chloramphenicol		Tetracycline		Streptomycin	
	P. O.	S. C.	P. O.	S. C.	P. O.	S. C.	P. O.	S. C.
Staphylococcus aureus Smith	0.3	0.3	Inactive 100.0	Inactive 100.0	5.2	6.0	—	—
Streptococcus pyogenes Group A	0.1	0.025	3.2	3.2	0.5	0.5	—	—
Salmonella typhimurium	18.0	12.8	310.0	250.0	62.4	59.2	—	—
Klebsiella pneumoniae	11.6	35.4	165.0	280.0	Inactive 400.0	61.0	—	—
Escherichia coli	21.0	8.1	27.0	50.0	36.5	5.6	38.5	1.8

Fig. 6 Serum concentrations in dogs following intramuscular administration of 5 mg/kg. Ampicillin (——), Methicillin (-·-·) and Benzylpenicillin (·····).

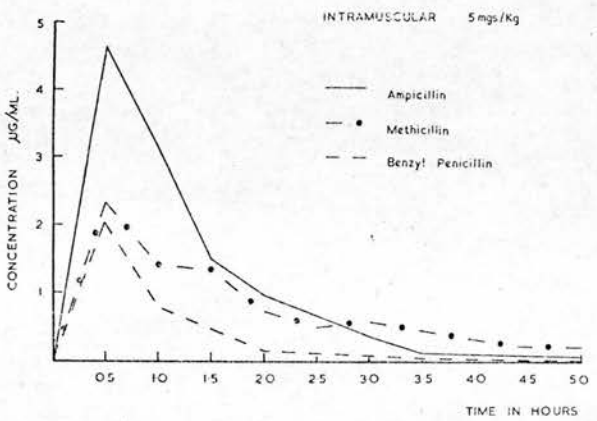


Fig. 7 Serum concentrations in dogs following oral administration of 20 mg/kg. Ampicillin (——) and Phenoxymethyl penicillin (·····).

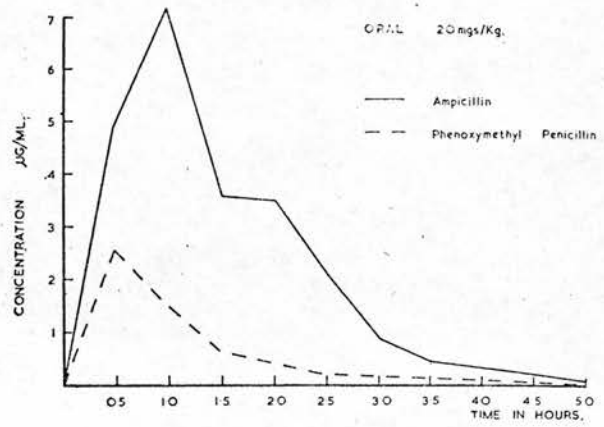


Table 2 Mean concentration $\mu g/g$ of methicillin and ampicillin appearing in the liver, kidney and serum of groups of ten rats after intramuscular administration of doses of 100 mg/kg of each penicillin.

Ampicillin 100 mg/kg Intramuscularly

Organ \ Time hrs.	Concentration $\mu g/g$ wet weight			
	0.5 hrs.	1.0 hrs.	2.0 hrs.	4.0 hrs.
Liver	175.0	70.5	5.8	1.0
Kidney	288.0	146.2	6.56	0.75
Serum	64.4	15.3	0.9	0.2

Methicillin 100 mg/kg Intramuscularly

Organ \ Time hrs.	Concentration $\mu g/g$ wet weight			
	0.5 hrs.	1.0 hrs.	2.0 hrs.	4.0 hrs.
Liver	32.8	23.8	12.9	4.1
Kidney	122.1	88.8	24.1	2.9
Serum	32.5	30.2	5.46	0.18

spaces.

Neither methicillin nor ampicillin readily penetrate into the C. S. F. but both drugs can be given intrathecally provided the administration is controlled. Excess concentrations of both antibiotics can cause convulsions in the same way as penicillin G. Both the new penicillins can penetrate into the aqueous humour, but to obtain the most satisfactory results the drugs have to be given subconjunctivally. In our experiments the penicillins were injected in 0.5 ml volumes subconjunctivally into the right eyes of a group of 6 rabbits. A volume of 0.5 ml saline was injected into the left eye and at 1, 3 and 4 hours after administration two animals from each group were killed and the eyes carefully dissected out. The aqueous humour was drawn asceptically from the anterior chamber. At the same time serum specimens were also collected and the concentration of the antibiotic in the fluids determined.

Elimination

In studying the excretion of an antibiotic, almost invariably only the urine excretion is considered. However, excretion in other secretory fluids are also to be taken into account, particularly in the bile. We have found, for example, that in the conscious rat 15% of methicillin can be eliminated in the bile. Allowing for the errors of assay when the total amount excreted in the urine and in the bile is calculated, nearly all of the administered methicillin can be accounted for. With methicillin the absorption from the gut is negligible and any of the antibiotic which is returned to the gut by the bile or saliva is completely lost to the body. On the other hand, the penicillins which are absorbed orally tend to be re-cycled and this factor alone can account for the more prolonged blood levels seen after the administration of orally absorbed penicillins.

With regard to the mode of elimination, ACRED *et al*[10,11] have shown that both methicillin and ampicillin are removed by the kidneys by renal tubular secretion and glomerular filtration in the same way as penicillin G, although it is much more difficult to block with probenecid the renal tubular secretion of ampicillin than the other two antibiotics. A quantitative study of the clearance rate of ampicillin has not yet been made, but should be of considerable interest to the pharmacologists.

Protein Binding

Finally, in considering the value of an antibiotic it is essential to appreciate the

Table 3 Concentrations of ampicillin and methicillin appearing in the aqueous humour and serum of groups of rabbits injected subconjunctivally in the left eye with 0.5 ml of a 25% solution of ampicillin or 50% solution of methicillin.

Penicillin \ Time	Concentration μg/ml											
	0.5 hrs.			1.0 hrs.			2.0 hrs.			4.0 hrs.		
	L	R	S	L	R	S	L	R	S	L	R	S
Methicillin	14.0	2.4	58.0	8.7	25.7	30.9	6.5	2.7	5.8	0.97	0.48	1.7
Ampicillin	60.0	0.7	12.3	48.0	0.8	5.6	19.0	0.3	0.4	1.1	0.4	0.1

L = Aqueous humour concentration μg/ml in injected eye.
R = Aqueous humour concentration μg/ml in control eye.
S = Serum concentration μg/ml.

significance of protein binding. It is generally assumed that protein binding leads to a decrease in antibacterial activity. Most studies have been carried out by static dialysis and assumptions on *in vivo* activity have been based on these *in vitro* results. However, the conditions in the body are vastly different and we feel that unless some information is available about the nature of the binding it is wrong to extrapolate from *in vitro* results activities *in vivo*. ACRED, BROWN, HARDY and MANSFORD[12] have studied protein binding on several penicillins by gel filtration, static and continuous dialysis techniques and have shown so far as the clinically active penicillins are concerned that the binding is very loose and that the penicillin is readily available. We have also shown that the concentrations of a penicillin which is bound to serum and another penicillin which is unbound to serum are the same or even greater in inflammatory fluids than in the serum. We injected glycogen, a non-chemotactic agent, intraperitoneally into two groups of rats, one which received 100 mg/kg ampicillin which is not protein bound, and the other which received 100 mg/kg of benzyl penicillin which is bound approximately 45% to serum proteins. At 0.5, 1, 2 and 4 hours afterwards 3 rats from each group were killed and the peritoneal fluid removed ; at the same time samples of serum were taken for assay. The concentrations of the antibiotics in the peritoneal fluid and the serum are given in Table 4. Apart from the first half an hour after administration when the concentration of the antibiotic in the peritoneal fluid was less than in the serum, the concentrations subsequently were $1\frac{1}{2}$ to 2 times as great as the serum. This is contrary to what would be expected if the penicillins were firmly bound to the serum proteins, and clear evidence that the penicillin-protein complex breaks down readily and allows the free penicillin to diffuse into the tissue spaces.

In conclusion, the discovery of new penicillins had led to two notable advances in the field of

Table 4 Concentrations of penicillin in peritoneal fluid and serum after intramuscular administration of 100 mg/kg penicillin G and ampicillin to groups of rats.

Penicillin	Fluid	Concentration μg/ml			
		0.5 hrs.	1.0 hrs.	2.0 hrs.	4.0 hrs.
Ampicillin	Peritoneal	50.0	24.1	3.4	0.9
	Serum	62.1	12.7	1.8	0.4
Benzyl Penicillin	Peritoneal	37.0	40.0	18.0	1.0
	Serum	53.0	26.0	12.0	7.8

chemotherapy. It is now possible to deal effectively with infections due to resistant staphylococci which have constituted a major problem in hospitals throughout the world. The introduction of oral penicillins effective against these organisms is a still further step towards combating these infections. The development of ampicillin has widened the clinical application of penicillins and much wider range of infections can now be successfully treated.

With these two additions in synthetic penicillins, the primary objectives of our research have been achieved but there is still considerable scope for the development of new penicillins. This applies particularly in the field of allergy where hypersensitivity to penicillins still remains a serious problem.

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A NEW APPROACH TO STUDYING THE PROTEIN-BINDING PROPERTIES OF PENICILLINS

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THE interaction of an antibiotic with serum is generally believed to result in a decrease in antibacterial activity. When the natural penicillins were originally examined, it was found that in the absence of serum, penicillin *K* was more active than penicillin *X*, and penicillin *X* was more active than penicillin *G*, but in the presence of serum, penicillin *G* was the most active. The reversal in activity was attributed to the fact that penicillin *G* was less bound to protein than penicillin *K* or *X*, and hence a greater concentration of free penicillin was available for antibacterial action[1].

The methods used to examine protein binding of penicillins have relied largely on *in vitro* static dialysis, but such methods, however, bear little relationship to the dynamic conditions in the body. We have, therefore, studied the phenomenon of protein binding with the view of determining more precisely its significance to antibacterial activity *in vivo*. Gel-filtration and both static and continuous dialysis methods have been used in this investigation.

The penicillins which were selected were penicillin *G*, 'Ampicillin' and '*BRL* 1071' (triphenylacetylpenicillin).

Static dialysis. To 5 ml. of human serum, 5 mg of the penicillin under examination were added. This was placed in a dialysis bag of 'Visking' tubing 0·75 in. diameter and suspended in 20 ml. normal saline and dialysed at 8° C for 24 h. Several dilutions of retentate and dialysate were prepared in M/20 phosphate buffer *p*H 7·0, and the amount of antibiotic was assayed by the cup-plate technique using *Sarcina lutea* as the test organism. The samples of each antibiotic were assayed against dilutions of known concentration of the same antibiotic.

Continuous dialysis. 5 mg of each antibiotic in 5 ml. serum were placed in 'Visking' tubing as described. The bags were suspended in a beaker through which water flowed continuously. The final concentration of the antibiotic was assayed in the serum at 24 h and the percentage obtained was calculated in terms of the concentration in serum at zero and 24 h.

Gel-filtration. 5 mg of each antibiotic were dissolved

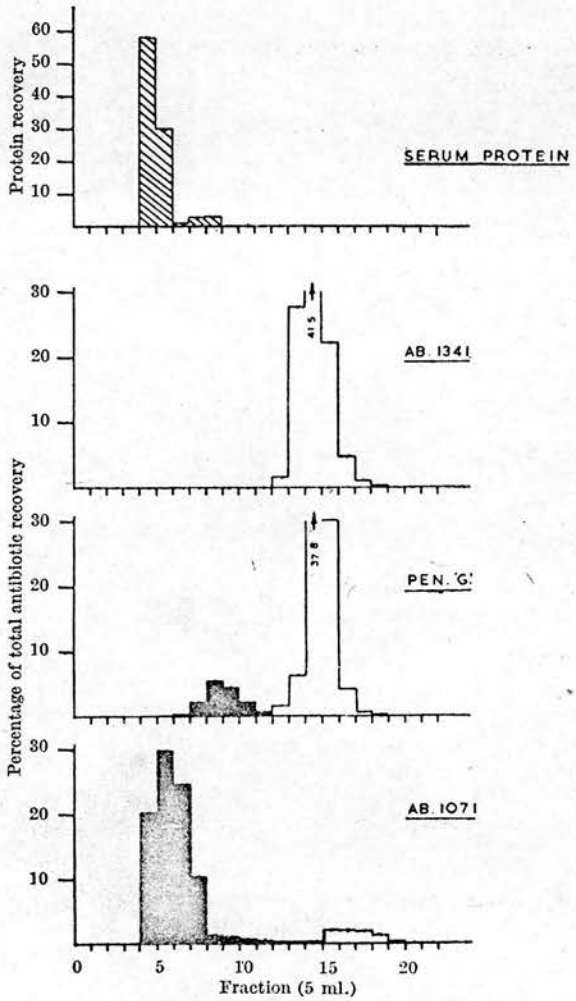

Fig. 1. Elution patterns from 'Sephadex' columns

in 5 ml. serum and allowed to stand for 18 h at 10° C. 3 ml. of the serum were applied to a column jacketed at 10° C and containing 100 ml. 'Sephadex *G*25' gel equilibrated with water, the flow rate being 20–25 ml./h. Fractions (5 ml.) were collected and protein elution was followed by optical density measurements at 280 mμ on a 'Unicam' *S.P.* 500. The fractions were assayed for antibiotic content. Aqueous solutions containing 1 mg/ml. of the antibiotics were similarly treated.

Protein fractionation. Further examination of the

bound fraction from the 'Sephadex *G*25' column was carried out using DEAE-'Sephadex *A*50'. After concentration by ultra-filtration the bound fraction was passed down a jacketed column kept at 10° C and containing 30 ml. of fully swollen DEAE-'Sephadex *A*50' equilibrated with 0·02 M phosphate buffer at *p*H 8·6. Stepwise elution was then carried out with 0·02 M phosphate buffer followed by saline of molarities 0·07, 0·12, 0·18 and 0·3 M, the change-over point being based on the optical densities of the fractions at 280 mμ.

Results

(*a*) *Dialysis.* The results obtained for the degree of protein binding are shown in Table 1. By static methods '*BRL* 1071' is bound 80–90 per cent to the serum proteins; penicillin *G* is much less bound, while 'Ampicillin' is only slightly bound. When examined by continuous dialysis, the final percentages bound were reduced but the reduction in the binding of '*BRL* 1071' was less than with the other penicillins (Table 1).

Table 1

Penicillin	Bound	
	Continuous	Static
Penicillin *G*	14·2 (5)	46·4 (21)
'*BRL* 1071'	41·4 (6)	91·6 (3)
'Ampicillin'	0·9 (3)	10·2 (6)

(*b*) '*Sephadex.*' Typical elution patterns from three penicillins of different binding properties obtained on 'Sephadex' columns are shown in Fig. 1. With the penicillin alone the antibiotic activity is confined to fractions 8–10. With serum alone the protein as determined by optical density at 280 mμ emerged in fractions 4–6. When the incubated penicillin serum mixture was passed down the 'Sephadex' column the protein again emerged in fractions 4–6, whereas the antibiotic activity showed two peaks, if there was significant binding. One peak was associated with the protein and the other again emerged in the protein-free fractions 8, 9 and 10. The degree of protein binding was then calculated from the antibiotic content of the appropriate fractions. The 'Sephadex' method[2] offers considerable advantages over dialysis methods since the fractionation occurs in less than 2 h, and after a short wash with water the column is ready for further use. The dilution factor of both the bound and unbound material is small, and this aids the sensitivity of the method. The reduction in time needed for the separation of bound and unbound compound is also an advantage when dealing with labile materials.

'Sephadex *G*25' grade has a lower limit for complete exclusion of molecular weights of 3,500–4,500 and is the preferred grade for protein binding studies. With the '*G*50' and '*G*75' grades of different degrees of cross-linkage, the protein peak is flattened and tends to tail into the unbound antibiotic peak.

(c) *Protein fractionation.* The bound fraction from the 'Sephadex *G*25' column, on further examination on DEAE–'Sephadex *A*50', gave the bulk of the antibiotic activity in the 0·18 M saline fractions, whereas aqueous penicillin solutions gave a peak of antibiotic activity in the 0·07 M saline fractions with some tailing into the 0·12 M saline fractions. Starch-gel and cellulose acetate electrophoresis was then carried out on the protein fractions. The material eluted with 0·02 M phosphate was found to consist of pure γ-globulin. The other fractions were all multi-component, but the 0·02 M, 0·07 M and 0·12 M fractions contained no albumin and were made up of mixtures of α- and β-globulins. The material eluted with 0·18 M saline consisted almost entirely of albumin whereas the material eluted with 0·03 M saline contained some material of the same mobility as albumin together with a high electrophoretic 'pre'-albumin content. It is evident from this method that the bound penicillin is associated with the albumin fraction of the serum protein.

Discussion

From the results of the three methods described, it is evident that penicillins can differ in the degree and nature of their binding to serum proteins. Static dialysis shows that 'Ampicillin' is slightly bound, penicillin *G* is moderately bound while '*BRL* 1071' is considerably bound.

The nature of the binding of '*BRL* 1071' and penicillin *G* is evident from the results under the different conditions of continuous dialysis and gel-filtration. The binding of penicillin *G* is shown to be of a very loose nature and the binding of '*BRL* 1071' to be very firm. Such differences in binding can affect activity *in vivo*. If a compound is firmly bound to the serum proteins the concentration of free penicillin is reduced and hence its intrinsic activity is reduced. The *in vivo* activity, however, may not be significantly influenced if the binding is of such a loose character that the penicillin is readily available for antibacterial activity. This is the case with penicillin *G* which is bound to the extent of 46 per cent to serum protein yet its activity is apparently uninfluenced by the presence of serum in the same way as a compound, for example, 'Ampicillin' which is only slightly bound. On the other hand, '*BRL* 1071' is very firmly bound and its activity is much reduced in the presence of serum *in vitro* and *in vivo*. Therefore, before conclusions on *in vivo* activity can be drawn from *in vitro* results, information should be available on both the actual degree and nature of the binding to serum proteins.

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Biotransformation of Hetacillin to Ampicillin in Man·

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Biotransformation of Hetacillin to Ampicillin in Man

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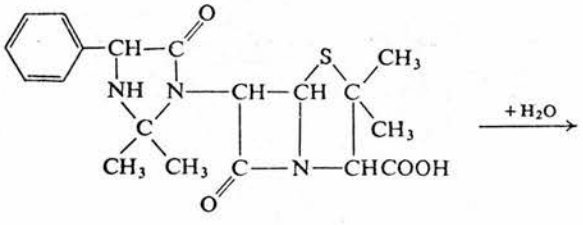
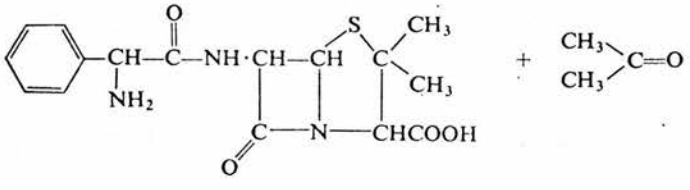
Biotransformation of Hetacillin to Ampicillin in Man. BROWN, D. M., HANNAN, D. P., and LANGLEY, P. F. (1969). *Toxicol. Appl. Pharmacol.* **15**, 136–142. A high voltage electrophoresis technique has been used to assay directly a mixture of two penicillins, ampicillin and hetacillin, in the plasma of humans after oral administration of hetacillin. Results show that ampicillin accounts for over 90% of the total biological activity at the peak plasma concentration, and it may be concluded that the clinical effectiveness of hetacillin is due to its conversion to ampicillin.

Hetacillin has essentially the same antibacterial spectrum of activity as ampicillin (Bunn *et al.*, 1965; Sutherland and Robinson, 1967), and it has been suggested that since hetacillin can readily be broken down to ampicillin *in vitro*, the activity of hetacillin *in vivo* also depends on the formation of ampicillin (Sutherland and Robinson, 1967).

Owing to the rapid hydrolysis of hetacillin to ampicillin (Fig. 1), it is impossible to obtain an estimate of the composition of mixtures of these antibiotics by the usual cup-plate assay method. We have therefore used a high voltage electrophoresis technique to separate and assay the antibiotics in the plasma following intramuscular administration to man.



Hetacillin (AB 2418)



Ampicillin (AB 1341) Acetone

FIG. 1. Hydrolysis of hetacillin.

METHODS

High voltage agar gel electrophoresis. The blood samples were assayed by high voltage agar gel electrophoresis as described by Lightbown and de Rossi (1965). A film of Noble agar,[1] approximately 2 mm thick, was prepared on a glass plate by pouring a 1% w/v solution of Noble agar in Tris-maleic acid buffer pH 5.6, into a mold. The plate was then placed on an aluminum block cooled to −15° and aqueous Tris-maleic acid buffer solution was placed in the electrode compartments, which were connected to the agar gel by gauze wicks. The samples were placed on the agar film with microcap disposable pipettes,[2] and a voltage of 1.5 kV was applied for 45 min in order to obtain separation of hetacillin and ampicillin.

On completion of electrophoresis, the agar film was overlaid with nutrient agar seeded with *Sarcina lutea*, and the plates were incubated overnight at 30°. The locations of the penicillins were seen as clear zones of inhibition on the plate.

Conversion of hetacillin to ampicillin on incubation in agar gel. Two microliter samples of hetacillin and ampicillin solutions, 0.25 mg/ml and 0.125 mg/ml, respectively, were subjected to electrophoresis on an agar film for 60 min; the agar film was then overlaid with nutrient agar seeded with *Sarcina lutea*.

A second plate was run under identical conditions to the first, but on completion of electrophoresis was not overlaid with seeded agar. Both plates were then incubated at 30° for 16 hours. After incubation, the second plate (test) was again subjected to electrophoresis under identical conditions to the first run and was then overlaid with seeded agar and incubated as previously.

Conversion of hetacillin to ampicillin in plasma. The effect of temperature on the rate of hydrolysis of a 5 μg/ml solution of hetacillin in fresh human plasma was studied. Hetacillin solutions, and control solutions containing 5 μg/ml ampicillin in plasma, were incubated at 37°, 20°, and 4° for 15, 60, 120, and 240 min and 24 hours. At each of these time intervals 10-μl samples of hetacillin and ampicillin incubates were subjected to electrophoresis, together with freshly prepared standard ampicillin solutions prepared from sodium ampicillin (potency 840 μg/mg) at concentrations of 5, 3, and 1.5 μg/ml plasma.

The zone diameters obtained from the standard solutions for each electrophoretogram were plotted against the logarithm of the concentration and, from the regression line obtained, the concentrations of the antibiotic in the test samples were estimated by interpolation.

Human plasma levels of hetacillin. Five volunteers in the nonfasting state were given an intramuscular dose of 500 mg of potassium hetacillin in 2 ml of sterile water, and blood samples were taken from the median cubital vein 10, 20, 40, 60, 80, and 160 min after dosing. The samples were placed in heparinized centrifuge tubes which had been precooled in ice, and they were then centrifuged at 1000 *g* for 5 min at 4°. The specimens were stored at this temperature until they were examined by the electrophoretic and cup–plate assay techniques.

Standard ampicillin solutions in plasma were prepared at concentrations of 2.5, 1.25, 0.63, 0.31, and 0.16 μg/ml. Forty-microliter samples of both the test plasma

[1] Difco Laboratories, Detroit, Michigan.
[2] Shandon Scientific Company Limited, 65 Pound Lane, Willesden, London, N.W.10.

samples and ampicillin standards were assayed by the electrophoretic technique. Total penicillin levels were also determined by the cup–plate assay method, using *Sarcina lutea* as the test organism.

These experiments were performed in duplicate on the same group of volunteers, a period of 3 weeks separating the first experiment from the second.

RESULTS

Hetacillin is rapidly hydrolyzed in aqueous solution. This is shown following the high voltage electrophoresis of a freshly prepared solution, where the presence of

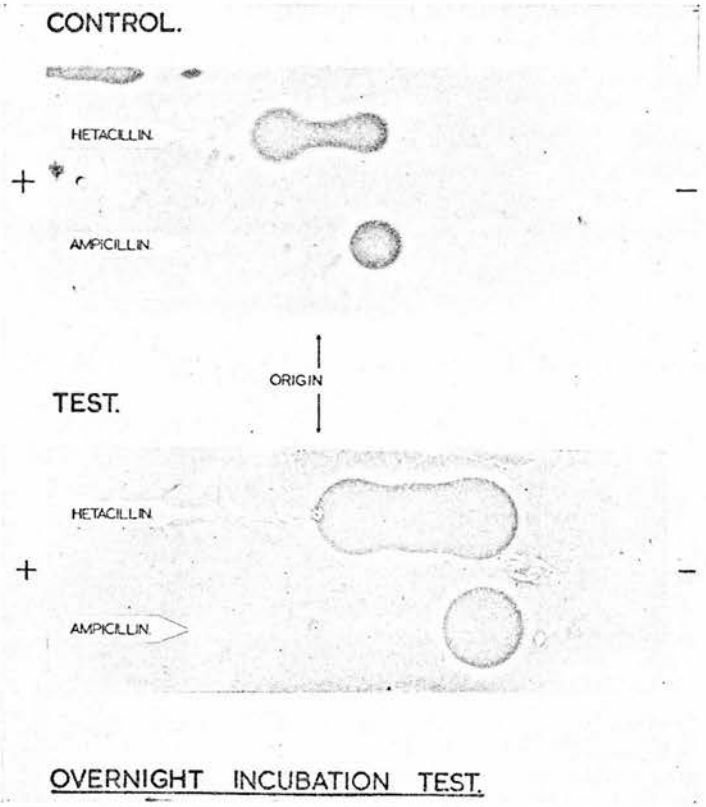


FIG. 2. Conversion of hetacillin to ampicillin after incubation in agar gel. "Control" indicates results obtained when samples were subjected to electrophoresis for 60 min and immediately overlaid with nutrient agar seeded with *Sarcina lutea*. "Test" indicates results obtained when samples similar to controls were subjected to a second electrophoresis after incubation at 30° for 16 hours but before being overlaid with seeded agar.

ampicillin is clearly demonstrated (Fig. 2, Control). The results of subjecting a hetacillin solution to high voltage electrophoresis, incubation of the plate for 16 hours followed by further high voltage electrophoresis are shown in Fig. 2 (Test). All the hetacillin has been converted to ampicillin. A comparison of equimolar solutions of

hetacillin and ampicillin by cup–plate assay demonstrated that both compounds gave rise to zones of the same diameter. In view of this and the fact that hetacillin is converted entirely to ampicillin during incubation on an agar gel plate, it is considered justifiable to assay hetacillin samples against ampicillin standards.

Conversion of Hetacillin to Ampicillin in Plasma

The hydrolysis of hetacillin in plasma at three different temperatures is shown in Fig. 3. Our results show that 50% hydrolysis of hetacillin occurs after approximately 25 min at 37°, 90 min at 20°, and more than 4 hours at 4°.

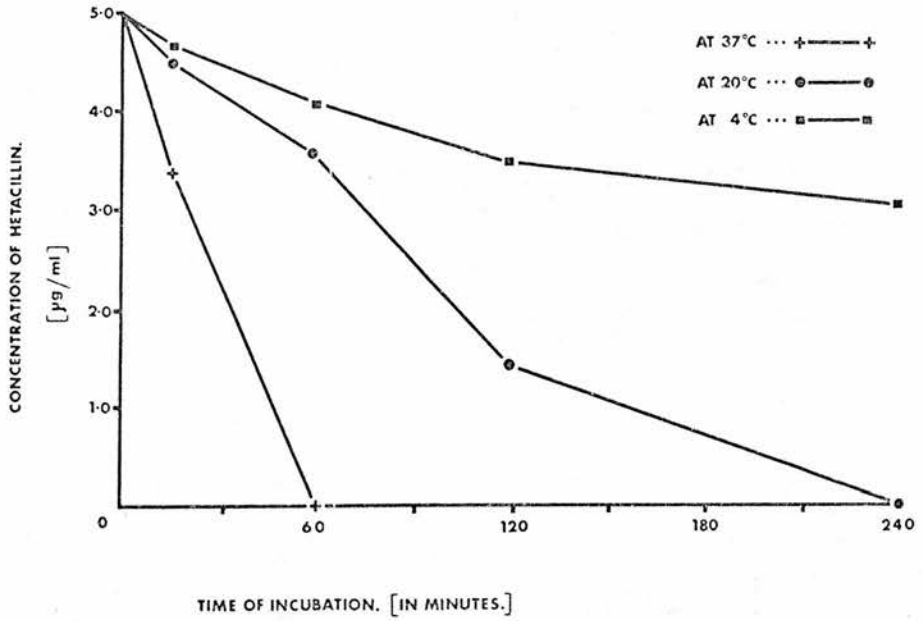


FIG. 3. Effect of temperature on *in vitro* hydrolysis of hetacillin in human plasma.

Plasma Levels in Man

The individual results for total antibiotic activity of hetacillin and ampicillin in the plasma following intramuscular administration of 500 mg of hetacillin are given in Tables 1 and 2.

The differences between cup–plate assay levels and total electrophoresis assay levels were first tested for significance at each time point, by the paired *t* test. Each time point was considered, where appropriate, for the two groups of 5 subjects and an overall group of 10 subjects. For the groups of 5, only one showed significance at the 5% level (160 min, group 2) and of the groups of 10, two showed significance at the 5% level (80 min and 160 min), i.e., no more than expected. It should be noted that the significant results were all associated with positive values and the higher blood levels. Nevertheless, no overall trend of the differences can be proved on the basis of these results. It is therefore assumed that the electrophoresis technique does not show any bias.

Having established the absence of bias, the ampicillin levels were calculated as a percentage of the total level found in the electrophoresis technique, and paired *t* tests for the two tests were carried out for each time point. Significance at the 5% level was found in only one instance (40 min). The mean percentage ampicillin levels are given in Table 3.

TABLE 1

CUP-PLATE ASSAY: LEVELS OF TOTAL PENICILLIN ACTIVITY IN HUMAN PLASMA AFTER INTRAMUSCULAR ADMINISTRATION OF 500 MG POTASSIUM HETACILLIN

Subject	Concentration (μg/ml)					
	10 min	20 min	40 min	60 min	80 min	160 min
			First experiment			
A	1.65	2.7	4.3	5.3	4.7	2.6
B	0.27	0.79	2.1	2.6	1.7	2.8
C	0.80	1.8	3.3	5.3	3.6	4.0
D	0.25	0.58	3.2	4.4	2.7	2.8
E	0.62	1.5	3.8	4.8	3.25	2.9
Mean	0.72	1.47	3.34	4.48	3.19	3.02
Mean total by electrophoresis	0.64	1.91	3.46	4.82	3.04	3.03
			Second experiment			
A	2.5	2.9	4.3	7.8	6.8	6.4
B	1.0	1.3	1.8	2.4	2.7	4.6
C	1.8	2.2	4.0	6.8	8.3	7.2
D	1.9	2.2	3.5	4.6	5.0	7.0
E	2.0	2.7	4.1	6.0	8.5	8.0
Mean	1.84	2.26	3.54	5.52	6.26	6.64
Mean total by electrophoresis	1.60	2.14	3.55	—	5.98	5.76

DISCUSSION

Sutherland and Robinson (1967) and Bunn *et al.* (1965) have reported on total levels of antibiotic activity following intramuscular administration of hetacillin, and although their results differ slightly both agree that the peak serum concentration occurs 2 hours after injection. In our studies the peak antibiotic level occurs somewhat earlier.

Sutherland and Robinson (1967) also reported that most of the antibiotic activity present in the blood was largely due to ampicillin, but no quantitative figures for this conversion were quoted. By using the high voltage electrophoresis technique to separate ampicillin and hetacillin in the plasma, we have assayed the respective concentrations of the antibiotics and have found that at the peak blood concentration of antibiotic, ampicillin could account for over 90%, and even 10 min after injection the conversion was as high as 68%. Therefore, it can be concluded that the clinical effectiveness of hetacillin is due to ampicillin.

TABLE 2

ELECTROPHORETIC ASSAY: LEVELS OF AMPICILLIN AND HETACILLIN IN HUMAN PLASMA AFTER INTRAMUSCULAR ADMINISTRATION OF 500 MG POTASSIUM HETACILLIN

	Antibiotic activity (μg/ml)																	
	10-min sample			20-min sample			40-min sample			60-min sample			80-min sample			160-min sample		
Subject	Amp.	Het.	Total	Amp.	Het.	Total	Amp.	Het.	Total	Amp.	Het.	Total	Amp.	Het.	Total	Amp.	Het.	Total
									First experiment									
A	0.64	0.47	1.11	2.2	0.81	3.01	3.5	0.57	4.07	5.0	0.94	5.94	4.0	0.23	4.23	2.8	Nil	2.8
B	0.18	Nil	0.18	0.54	0.12	0.66	2.1	0.26	2.36	3.0	Nil	3.0	1.7	Nil	1.7	2.7	0.05	2.75
C	0.62	0.29	0.91	2.1	0.78	2.88	3.0	0.50	3.50	4.6	0.52	5.12	3.4	0.20	3.6	4.3	0.16	4.46
D	0.20	Nil	0.20	0.60	0.13	0.73	3.1	0.41	3.51	4.1	0.47	4.57	2.25	Nil	2.25	2.3	Nil	2.3
E	0.52	0.30	0.82	1.41	0.72	2.13	3.3	0.57	3.87	4.6	0.68	5.28	3.0	0.25	3.25	2.8	Nil	2.8
Mean	0.43	0.21	0.64	1.37	0.51	1.88	3.0	0.46	3.46	4.26	0.52	4.78	2.87	0.14	3.01	2.98	0.04	3.02
									Second experiment									
A	1.30	0.74	2.04	2.10	0.80	2.90	3.82	0.81	4.63	—	—	—	5.70	0.60	6.30	5.60	0.20	5.80
B	0.31	0.26	0.57	0.75	0.35	1.10	1.25	0.47	1.72	—	—	—	2.60	0.05	2.65	3.40	Nil	3.40
C	0.95	0.62	1.57	1.50	0.71	2.21	3.10	0.75	3.85	—	—	—	7.20	0.12	7.32	5.80	0.17	5.97
D	1.10	0.86	1.96	1.30	0.65	1.95	2.75	0.62	3.37	—	—	—	4.80	0.11	4.91	5.90	0.13	6.03
E	1.15	0.74	1.89	1.69	0.84	2.53	3.32	0.91	4.23	—	—	—	7.50	0.05	7.55	7.50	0.05	7.55
Mean	0.96	0.64	1.61	1.47	0.67	2.14	2.85	0.71	3.56	—	—	—	5.56	0.19	5.75	5.64	0.11	5.75

TABLE 3

PARAMETERS FOR THE MEAN PERCENTAGE AMPICILLIN LEVEL BASED ON 10 RESULTS (5 RESULTS IN THE CASE OF 60 MIN)

Time (min)	Mean	Standard error
10	68.5	5.4
20	71.8	1.9
40	83.0	1.6
60	90.2	2.7
80	96.5	1.1
160	98.6	0.5

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TISSUE DISTRIBUTION OF PENICILLINS

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WHILE it is of obvious importance to determine the antibacterial activity of a penicillin in the blood it is perhaps of greater importance to know what concentrations are present in the extravascular fluids. In view of the current interest in protein binding and its influence on activity it is also of importance to determine in what way binding influences distribution throughout the body.

Usually methods for estimating tissue levels of antibiotics have consisted mainly of grinding the tissue or organ in a homogeniser with the appropriate buffer, spinning down the debris and estimating the penicillins in the supernatant. The total amount of penicillin present is calculated and then expressed as the amount per gram of tissue, wet or dry weight. However these figures give very little information about the actual tissue levels. Depending on the vascularity of the organs they may reflect largely the blood levels. Alternatively, they may give quite erroneous results due to specific concentration within the organ, for example, the high levels found in the liver and kidney merely reflect the high concentration in the bile and urine. It is highly improbable that there is specifically high concentration of the antibiotics in the intracellular space of the liver and kidney. Hence it is very difficult to get a true figure for the amount of penicillin in tissues. We therefore estimated the concentration of penicillin in inflammatory fluids and lymph which can be obtained in adequate quantities for assay purposes. In addition we determined the penicillin concentration in granuloma tissue. For the studies, four penicillins were selected—phenethicillin, phenoxymethylpenicillin, cloxacillin and ampicillin.

In order that all the results should pertain to the one species the serum protein binding was determined for each using rat serum by the ultrafiltration technique. The assay procedure was the cup plate technique using *Sarcina lutea* as the test organism, the usual precautions necessary for assaying in the presence of protein being observed. (Table I).

TABLE I.

Binding of the Listed Penicillins to Rat Serum

Penicillin	% Bound
Ampicillin	5.2
Cloxacillin	81.5
Phenoxymethylpenicillin	77.4
Phenethicillin	84.3

Since it is generally agreed that penicillin combines reversibly only with the albumin fraction of tissue protein, it was also of interest to see if differences in the albumin content of the body fluids could influence the penetration from serum. We therefore estimated the total amount of protein by the micro-biuret method in the autoanalyser and the albumin/globulin ratios were determined by electrophoresis. The results are given in Table II.

TABLE II

Sample	Total Protein gms. %	Albumin/ Globulin Ratio
Rat Lymph.	3.2	1.33:1
Rat Peritoneal Fluid.	6.8	1.27:1
Rat Serum.	6.8	1.2 :1

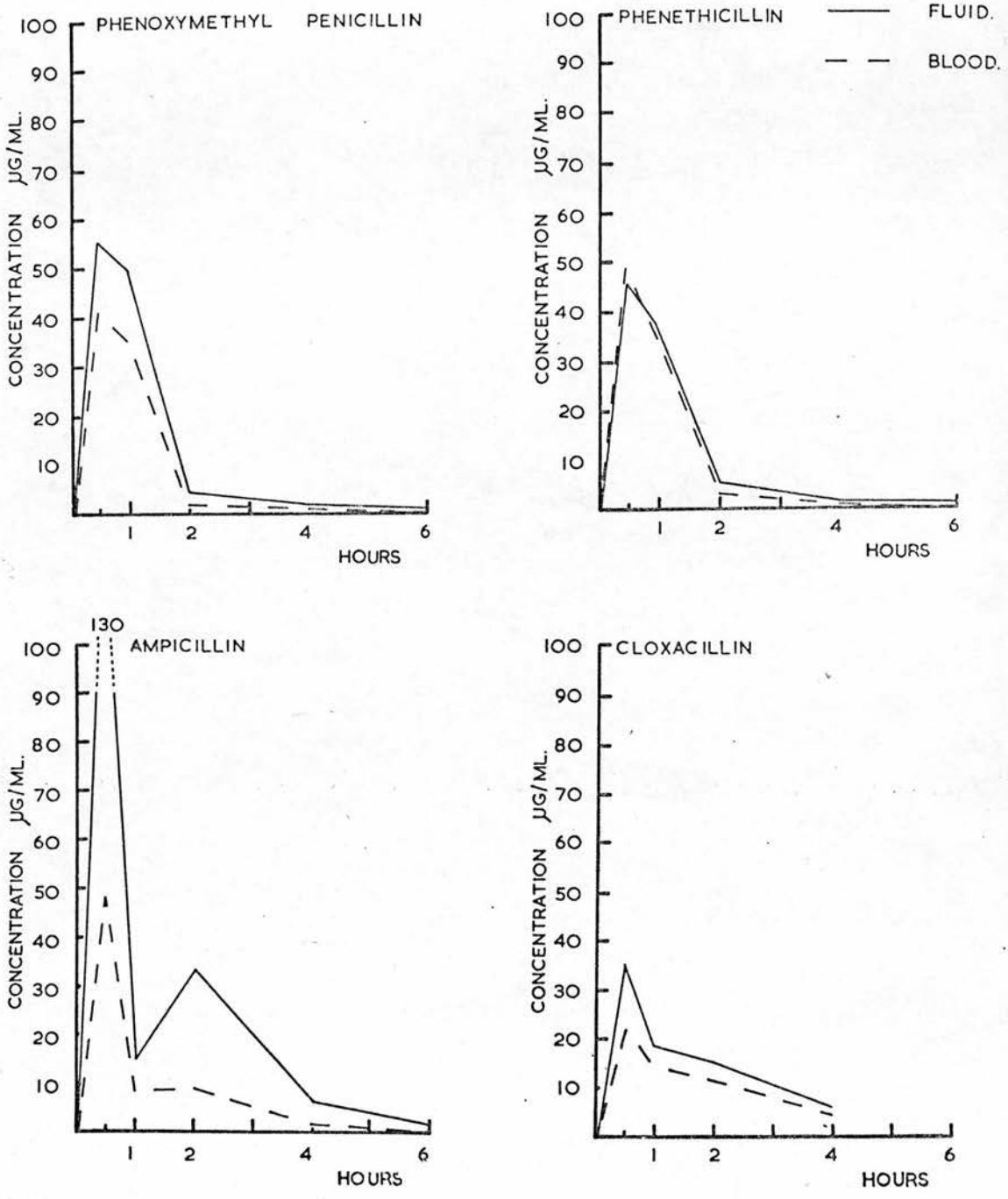

FIG. 1.—Concentrations of Penicillins µg./ml. in Blood and Inflammatory Peritoneal Fluid of Rats following the Intramuscular Administration of 100 mg./kg. of the Penicillins.

To estimate the concentration of penicillin in inflammatory fluids groups of ten rats of average weight 150 gms., ten per group, were injected intraperitoneally with 0.1 ml. turpentine. Immediately afterwards the penicillin being investigated was injected intramuscularly into the left hind limb at a dose of 100 mg./kg. At ½, 1, 2, 4 and 6 hours afterwards the rats were killed and the peritoneal fluid withdrawn, a blood sample was also removed, and assayed. The fluid was diluted with M/20 phosphate buffer pH 7.2 for assay. The results are shown in Figure 1.

The serum and inflammatory fluid concentrations are similar with phenethicillin whereas the inflammatory fluid concentrations are slightly higher than the blood levels with phenoxymethylpenicillin and cloxacillin. However ampicillin gives much higher levels in the inflammatory fluid than the blood. Irrespective of the degree of protein binding one would expect equal concentrations of antibiotics on either side of the capillary wall provided one assumes that the capillaries function as semi-permeable membranes and the concentrations of protein in the serum and intraperitoneal fluids are the same. Since the total concentration of ampicillin is much greater in the inflammatory fluid than the serum, simple equilibrium conditions do not appear to exist, and the concentrations found would not appear to be entirely dependant on the degree of protein binding.

Further evidence that the degree of binding to serum does not appear to influence penetration into extravascular tissues was obtained by studying the penetration of penicillins into granuloma tissue. A cotton pellet was inserted into the subcutaneous tissues in the axillary region of rats and 4-5 days afterwards when the granuloma around the pellet had formed, injections of 100 mg./kg. of the penicillin were given intramuscularly. The rats were killed at ½, 1, 2, 4 and 6 hours afterwards. The granuloma was removed, homogenised with M/20 phosphate buffer pH 7.2 and the penicillin assayed in the homogenate. A blood sample was taken at each time period. Figure 2 shows that in general the concentration per gram granuloma is less than the blood concentration but there is a considerable amount of fibrous tissue present into which the penicillin cannot penetrate. However the amounts of phenoxymethylpenicillin, phenethicillin and cloxacillin present were similar in relation to the blood levels but ampicillin which is the least protein bound of the four penicillins penetrates much less readily.

Finally we estimated the concentration of penicillin in lymph. Werwey and Williams (1962) have shown that lymphatic levels during continuous intravenous infusion are in general less than the corresponding serum levels, but their method of administration is not one which is normally employed clinically. We therefore decided to study lymphatic levels following intramuscular administration. Groups of five rats were anaesthetised with pentobarbitone and the thoracic lymph duct cannulated. The penicillins were injected intramuscularly into the left hind limb. Samples of lymph and blood, which was taken from the tail vein, were removed for assay at ½, 1, 2 and 4 hours. The levels obtained are given in Figure 3.

With phenoxymethylpenicillin, phenethicillin and cloxacillin the levels in the lymph are somewhat higher than those in blood while the levels of ampicillin in lymph are much greater than the corresponding blood levels. It could be argued that since ampicillin is only slightly protein bound that it diffuses more rapidly from the site of injection than the other penicillins which are more heavily bound. It is then taken up in the lymph in much greater concentration. Our experiments with inflammatory fluids and granuloma tissue would appear to show however that the combination of penicillin and protein does not influence the passage of the penicillin from one site to another.

The albumin content of the peritoneal fluid is slightly higher than serum while the content of the lymph is much less even though the albumin/globulin ratio is increased. It would seem therefore that the higher total levels in lymph and peritoneal fluid than blood are not a consequence of an increased amount of albumin in the tissues.

These results also suggest that the body is not divided into a number of compartments separated by semi-permeable membranes but rather the division between each compartment allows particular molecules to pass through selectively. In other words it is possible to obtain different concentrations in different tissues irrespective of the albumin content on either side of the dividing membrane.

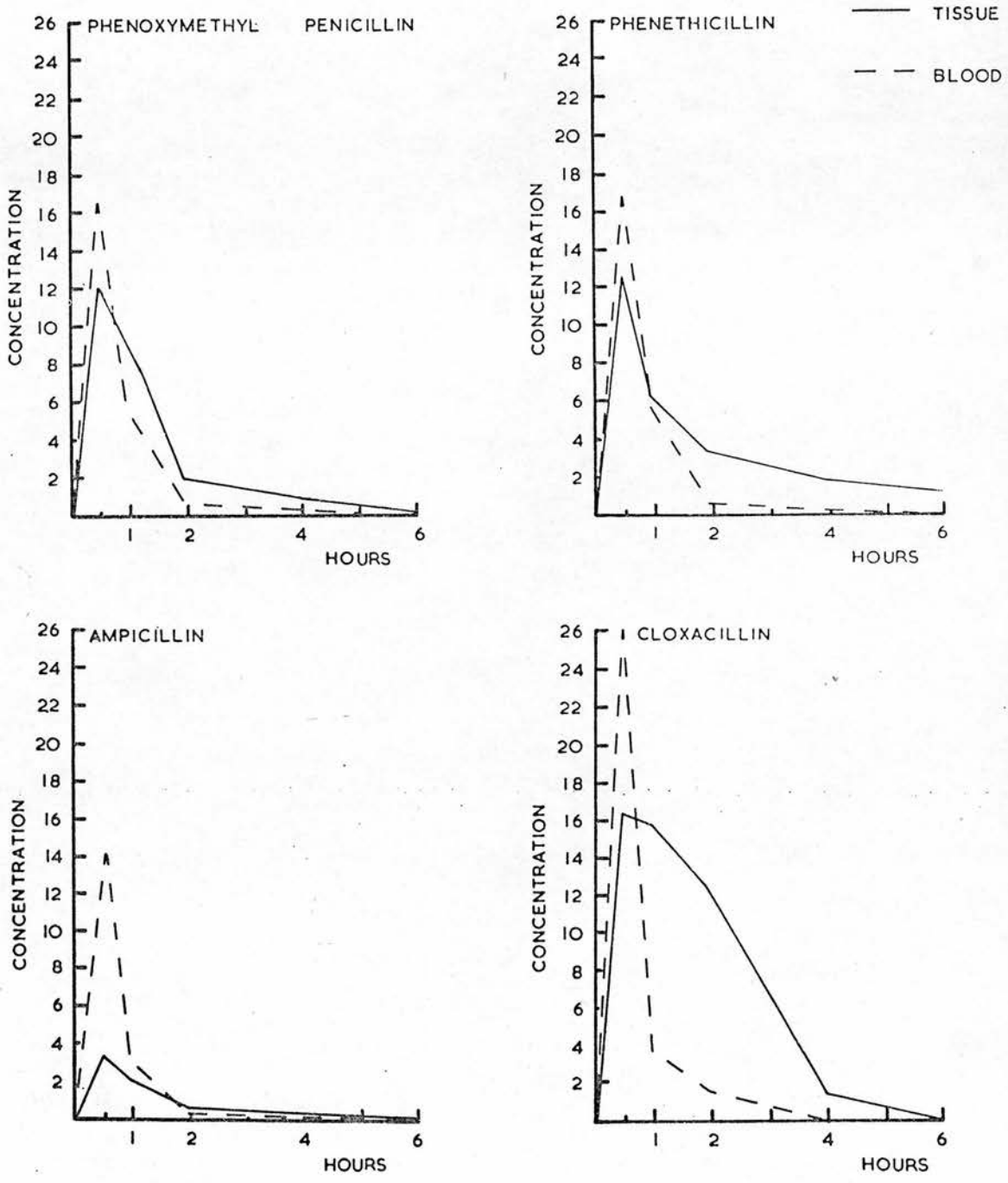

FIG. 2.—Concentrations of Penicillins in Granuloma Tissue μg./gm. and Blood μg./ml. following Intramuscular Administration of 100 mg./kg. of the Penicillins to Rats.

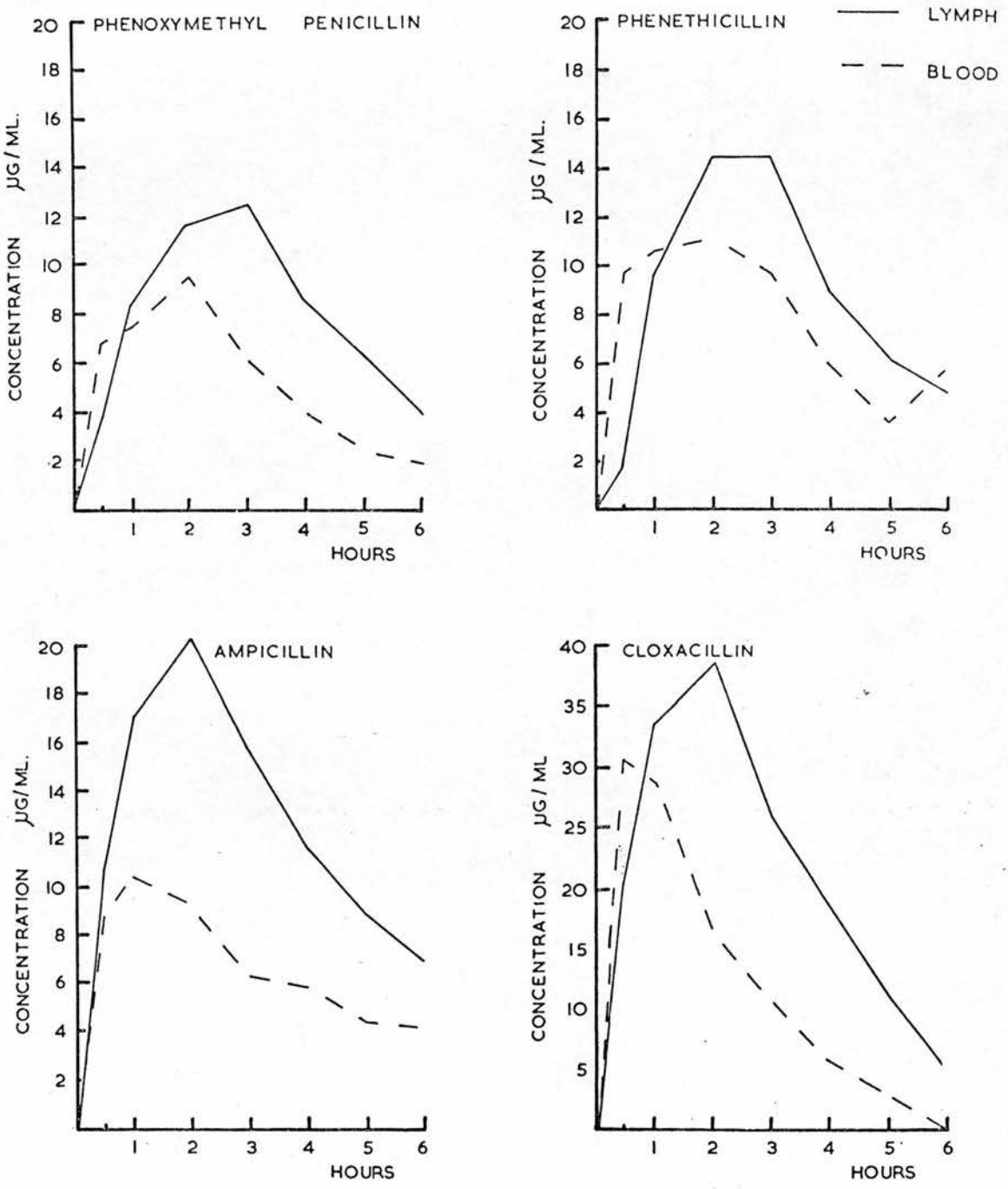

FIG. 3.—Concentrations of Penicillins µg./ml. in Blood and Lymph following Intramuscular Administration of 100 mg./kg. of the Penicillins to Rats.

When comparing the properties of different penicillins with a view to predicting their therapeutic efficacy it is not only necessary to know the concentration of penicillin in the blood and the degree of binding to the serum albumin, it is also necessary to know the levels in the extravascular fluids and the degree of binding to the albumin in these fluids.

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THE DISTRIBUTION OF ANTIBACTERIAL AGENTS BETWEEN PLASMA AND LYMPH IN THE DOG

BY

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The distribution of antibacterial agents between plasma and lymph in the dog

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Summary

1. Plasma, peripheral and thoracic lymph concentrations of penicillin V, phenethicillin, carbenicillin, ampicillin, cloxacillin, penicillin G, chloramphenicol and sulphadiazine were determined at various time intervals up to 6 h following intramuscular administration of 50 mg/kg to dogs.

2. Peak plasma concentrations of the penicillins occurred within half an hour after administration with the peak lymphatic concentrations occurring 1·5 to 3 h afterwards. For the remaining period of the test the concentration in the lymph exceeded the corresponding concentration in the plasma. Sulphadiazine gave concentrations in thoracic lymph equal to the plasma concentration, but the peripheral lymph concentrations were lower while the concentrations of chloramphenicol in both peripheral and thoracic lymph were always lower than the plasma concentrations.

3. After the peak concentrations were reached, the concentration curves for penicillins in lymph followed the same pattern as found in plasma, the penicillin concentrations declining exponentially. Sulphadiazine produced more persistent levels both in lymph and in plasma while the concentrations of chloramphenicol were still rising 6 h after administration.

4. The free concentrations of penicillin in lymph were equal to the free concentrations in plasma, whereas the concentrations of free sulphadiazine and chloramphenicol in lymph were less than those in the plasma.

Introduction

Since bacterial infections are normally localized and confined to specific sites within the body, it is perhaps of greater importance to know the concentrations of antibiotic at these sites rather than the corresponding blood levels. In addition, since it is generally agreed that the protein bound fraction of the antibiotic is inactive the concentration of the free antibiotic in the extravascular fluids should also be known. There have been only a limited number of studies carried out to determine free antibiotic levels in infected tissues because it is technically difficult to get sufficient fluid from such a site to carry out the assays. According to Yoffey & Courtice (1956) there are only minor differences in the composition of peripheral lymph and interstitial fluid from which it is derived. We have therefore carried out experiments to determine the relationship between plasma and peripheral lymph

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concentrations of some antibacterial agents and have noted the effect of serum protein binding on the distribution in blood and extravascular tissues.

Methods

Drugs

The following chemotherapeutic agents were used: cloxacillin (Orbenin, Beecham Research Laboratories) was used as the sodium salt, phenethicillin (Broxil, Beecham Research Laboratories) as the potassium salt, phenoxymethylpenicillin (Penicillin V, Eli Lilly & Co. Ltd.) as the potassium salt, ampicillin (Penbritin, Beecham Research Laboratories) as the sodium salt, benzylpenicillin (Crystapen, Glaxo Laboratories Ltd.) as the sodium salt, carbenicillin as the sodium salt (Pyopen, Beecham Research Laboratories), chloramphenicol (Chloromycetin, Parke Davis & Co.) and sulphadiazine (May & Baker Ltd.).

Solutions of the penicillins were prepared in normal saline. Chloramphenicol solutions were prepared by dissolving the compound in absolute ethanol and making up to volume with normal saline. The resulting solution contained 10% ethanol. The solution of the sulphonamide was prepared by dissolving the agent in 1 N NaOH, neutralizing with 1 N HCl and making up to volume with normal saline.

Doses

Groups of two or three greyhounds (20–30 kg body weight) were used. The compounds were administered intramuscularly into the left hind leg muscle at a dose of 50 mg/kg.

Samples of blood and lymph for assay were taken at 0·5, 1, 2, 3, 4, 5 and 6 h following administration. All samples were stored at 4° C until assayed.

Assays

Penicillins and chloramphenicol were determined in the samples by the hole-plate technique ; *Sarcina lutea* was used as the test organism. The plates were incubated overnight at 29° C. In the assays in which the samples were used undiluted, the solutions for the standard curve were prepared in the same fluid as the test specimen, blood or lymph. When specimens required dilution they were assayed against the standard prepared in saline. The zone diameter (mm) of each standard solution was plotted against the logarithm of the concentration (μg/ml) and from the regression line obtained the concentration of the antibiotic in the sample was estimated by interpolation. Concentrations appearing in plasma were calculated assuming a packed cell volume of 40%. Studies previously carried out had indicated that none of the compounds used in these experiments penetrated red cells or were bound to cells.

The free and total sulphonamide content of the sample was determined using the colorimetric method of Bratton & Marshall (1939).

Protein content

The total protein content (g/100 ml) of lymph and serum was determined by the biuret method. The albumin/globulin ratio was determined by the cellulose acetate electrophoresis technique. 10 μl quantities of the samples were spotted onto the

cellulose strips and a constant current of 0·6 mA/cm width was applied for 3 h. The buffer used was 0·06 M barbitone, pH 8·6. The strips were stained with Ponceau "S" (0·2% in 3% trichloracetic acid) and were scanned by transmitted light using a Chromoscan densitometer.

Ultrafiltration

The amount of binding of the compounds to proteins was determined by ultrafiltration. Samples of lymph and serum were obtained from a number of dogs and pooled. The pooled samples, stored at 4° C, were kept for a maximum period of 2–3 days before use. The pH of the samples was checked before each experiment and where necessary adjusted to pH 7·2–7·4 by bubbling CO_2 through the specimen.

Concentrations of the chemotherapeutic agents of 40 μg/ml were prepared in both serum and lymph, and 2·0 ml samples were placed into 8/32 inch Visking tubing which was suspended in a tube 3 inches × 1 inch. The pressure in the tube was reduced to 15 mmHg (1 mmHg≡1·333 mbar) and the system was sealed to prevent evaporation of the filtrate. The filtrate volume was never allowed to exceed 10% of the original solution for filtration. Control experiments were carried out with aqueous solutions to confirm that free passage of the compounds through the membrane occurred in the absence of protein. All experiments were carried out at room temperature (20°–24° C) over a maximum period of 90 min. The filtrate was assayed and the binding of the compounds to serum and lymph was calculated using the following formula:

$$\%\text{binding} = \frac{\text{Initial concentration } \mu\text{g/ml} - \text{filtrate concentration } \mu\text{g/ml} \times 100}{\text{Initial concentration } \mu\text{g/ml}}$$

Collection of lymph

The method used for the collection of peripheral lymph was similar to that described by Smith, Dunton, Protas, Blocker, Cooley & Lewis (1959) and Verwey & Williams (1962a, b).

The dogs were anaesthetized intravenously with pentobarbitone sodium (veterinary Nembutal) 30 mg/kg. The right hind paw lymphatics and the thoracic duct were cannulated as follows:

(*a*) *Peripheral lymph.* The skin on the dorsal surface of the paw was shaved and an area 5 × 2·5 cm carefully removed from a region extending forward from the base of the metatarsals. To render the lymphatics clearly visible 0·1 ml of Evans Blue dye (0·1%) was injected into the subcutaneous spaces between the metatarsals. About 1 cm of one of the larger paw lymphatic vessels was dissected free and cannulated with a No. 12 hypodermic needle (23 gauge) from which the mount had been removed. It was carefully secured and a length of polythene tubing (Portex polythene No. 46, 0·8 mm external diameter) was then attached. The lymph was collected in tubes containing 20 i.u. of heparin. Collection from the peripheral lymph vessel on the side of the animal opposite the injection site was carried out since the concentration of the antibacterials in the lymph on the injected side could be influenced by drainage from the site of injection. Volumes of peripheral lymph up to 1 ml/h were collected.

(*b*) *Thoracic lymph.* A median longitudinal incision through the skin of the ventral surface of the shaved neck of the dog was made, the trachea and left carotid artery exposed, and a respiratory cannula inserted. At the junction of the subclavian, jugular and common jugular veins the anastomosis of the lymphatic system with the venous system was located. At the anastomosis, lymphatic vesels from the head and left foreleg meet together with the thoracic lymph duct, which ascends from the thoracic region and is identified as a flattened thin walled translucent vessel. The duct was carefully freed from the surrounding tissues and cannulated with a polythene cannula 1·7 mm external diameter. Lymph which flowed freely along the duct was collected in tubes containing heparin (0·05 ml, 1,000 i.u./ml). A mean thoracic lymph flow of 23·6 ml/h was produced.

Results

Protein concentration

The total protein content of serum, thoracic and peripheral lymph in g/100 ml and the albumin globulin ratios are given in Table 1. The electrophoretic studies showed that all the plasma proteins were present in the lymph, but at lower concentrations. The lymph values are in good agreement with reported results (Field, Leigh, Heim & Drinker, 1934–1935 ; Glenn, Gresson, Bauer, Goldstein, Hoffman & Healey, 1949 ; Nix, Mann, Bollman, Grindlay & Flock, 1951 ; Courtice & Morris, 1955 ; Verwey & Williams, 1962a, b).

Protein binding

The degree of binding of the antibacterial agents to serum and lymph proteins is shown in Table 2.

The binding to the serum protein decreased in the following order: phenethicillin, cloxacillin, penicillin V, chloramphenicol, penicillin G, sulphadiazine, carbenicillin and ampicillin. The amount of drug bound to protein in the serum, thoracic and peripheral lymph depended on the protein content of these fluids. The decrease

TABLE 1. *Total protein content (g/100 ml) and albumin globulin ratios of dog serum, thoracic and peripheral lymph*

Fluid	Total protein (g/100 ml)	Albumin/globulin ratio	Calculated albumin concentration (g/100 ml)
Thoracic lymph	3·1	1·3:1	1·8
Peripheral lymph	1·47	0·96:1	0·7
Serum	5·7	1:1	2·8

TABLE 2. *Percentage of drug bound to protein in serum, thoracic and peripheral lymph, as determined by ultrafiltration*

Compound	Serum	Thoracic lymph	Peripheral lymph
Phenethicillin	65·5	58·0	11·1
Cloxacillin	64·5	59·2	36·9
Penicillin V	63·9	55·7	6·3
Penicillin G	33·4	34·1	21·6
Carbenicillin	4·7	0	0
Ampicillin	3·5	0	0
Chloramphenicol	36·1	36·8	21·0
Sulphadiazine	21·7	23·0	6·4

in the binding in the peripheral lymph is therefore due to the lower protein content. The results obtained for the penicillins are in good agreement with those reported by Verwey & Williams (1962b). The percentage protein binding of sulphadiazine to dog serum is in close agreement with that reported by Anton (1960).

Concentration of antibacterial agents in plasma, peripheral and thoracic lymph

The total concentrations of the antibacterial agents appearing in the three body fluids following intramuscular dosing are shown in Table 3. The free (unbound) concentrations (Table 4) were calculated by deducting from the total concentration the fraction bound as estimated by the ultrafiltration experiments.

Since two to three animals were used for each estimation, only the mean antibacterial concentrations have been quoted. The majority of individual values were within 20% of the mean, but a few values varied by as much as 50%. Within these limits the penicillins gave similar concentration patterns in the plasma, thoracic and peripheral lymph, the peak plasma concentration occurring 0·5–2 h after dosing, and the peak lymphatic concentration occurring 1–3 h after dosing. Following the peak values the total thoracic and lymphatic concentrations for the penicillins and sulphadiazine were of the same order as the plasma. The total plasma concentration for chloramphenicol, however, was greater than the lymphatic concentration and did not follow the same pattern as that of the penicillins. The concentration with this antibiotic was still increasing 6 h after the time of administration.

The concentration of free penicillin in the lymph was generally in excess of the plasma following the peak plasma concentration. The free levels of sulphadiazine in thoracic lymph were of the same order as those found in plasma, but the free concentrations in peripheral lymph were less. The free concentrations of chloramphenicol in peripheral and thoracic lymph were less than the plasma concentration.

Discussion

Estimates of the amount of drug passing from the blood into the tissues have in many previous studies been made by determining drug concentrations in macerated organs. The results obtained by this method can be misleading, however, owing to the presence of blood in the tissue, and therefore the apparent concentration of antibiotic in the tissue can vary with the vascularity of the particular tissue. Extravascular tissue fluids are, however, difficult to obtain in sufficient quantities for assay, but Yoffey & Cortice (1956) consider that peripheral lymph, which can be obtained in adequate quantities uncontaminated by blood, is very similar to extravascular fluids. Schachter (1948) was the first to determine the concentration of penicillin G in the lymphatic system of dogs following intramuscular and intravenous administration. He found that penicillin persisted in the thoracic lymph longer than in the blood, but that the peak concentration following intramuscular dosing was less than in blood. A more recent study was made by Verwey & Williams (1962a, b), who determined the concentrations of a number of penicillins in peripheral lymph during constant intravenous infusion in dogs. These authors found that the amounts of the penicillins free in lymph were very similar to the amounts free in plasma and

TABLE 3. *Mean total concentration (μg/ml) of antibacterials appearing in plasma, thoracic and peripheral lymph of dogs (groups of two or three) following intramuscular administration of 50 mg/kg*

Fluid	Compound	Concentration (μg/ml) at hours after administration						
		0·5	1·0	2·0	3·0	4·0	5·0	6·0
Plasma	Cloxacillin	16·7	11·7	7·8	6·0	4·5	4·0	3·3
	Phenethicillin	8·0	13·2	14·0	11·0	8·8	6·7	6·2
	Penicillin V	8·7	7·0	7·2	6·5	4·3	3·2	2·7
	Penicillin G	58·7	65·8	39·2	22·5	15·7	10·5	5·0
	Carbenicillin	70·25	43·0	18·5	7·2	5·5	3·25	0
	Ampicillin	14·7	15·8	12·5	9·2	5·2	4·0	3·5
	Chloramphenicol	10·7	10·0	11·8	15·3	14·3	14·2	20·0
	Sulphadiazine	19·2	16·7	16·6	20·0	16·7	14·7	—
Peripheral lymph	Cloxacillin	0·7	3·6	6·3	4·7	2·5	2·8	1·7
	Phenethicillin	1·9	5·0	8·2	8·0	4·7	4·1	3·8
	Penicillin V	0·9	2·7	3·7	4·5	3·4	2·4	1·8
	Penicillin G	8·9	33·0	37·5	22·5	16·7	11·0	4·2
	Carbenicillin	4·5	25·5	32·5	14·7	6·9	5·8	—
	Ampicillin	4·4	9·7	10·6	8·9	8·7	5·6	—
	Chloramphenicol	2·3	6·5	6·7	6·5	7·6	11·8	14·0
	Sulphadiazine	2·0	3·0	8·0	14·0	13·0	10·0	4·0
Thoracic lymph	Cloxacillin	4·8	11·1	9·3	6·8	7·2	3·2	2·5
	Phenethicillin	0·6	5·4	11·5	13·8	11·9	7·9	7·1
	Penicillin V	1·2	7·8	10·0	6·6	5·0	3·3	1·8
	Penicillin G	23·5	46·5	31·5	27·0	18·6	11·2	6·9
	Carbenicillin	15·5	68·0	50·0	21·75	6·3	4·6	4·3
	Ampicillin	5·3	17·2	19·3	12·9	9·2	6·8	6·7
	Chloramphenicol	2·5	6·0	7·7	8·9	9·3	8·7	13·0
	Sulphadiazine	5·5	16·5	21·5	16·5	19·0	21·3	18·8

TABLE 4. *Calculated free concentrations (μg/ml) of antibacterials in dog plasma, peripheral and thoracic lymph*

Fluid	Compound	Concentration (μg/ml) at hours after administration						
		0·5	1·0	2·0	3·0	4·0	5·0	6·0
Plasma	Cloxacillin	5·9	4·2	2·8	2·1	1·6	1·4	1·2
	Phenethicillin	1·3	4·6	4·8	3·8	3·0	2·3	2·1
	Penicillin V	3·1	2·5	2·6	2·4	1·6	1·2	0·97
	Penicillin G	39·1	43·8	28·2	15·0	10·5	7·0	3·4
	Carbenicillin	66·9	40·9	17·6	6·8	5·2	3·1	0
	Ampicillin	14·2	15·2	12·1	8·9	5·0	3·9	3·4
	Chloramphenicol	6·8	6·4	7·55	9·8	9·15	9·1	12·8
	Sulphadiazine	15·0	13·1	13·0	15·7	13·1	11·5	—
Peripheral lymph	Cloxacillin	0·4	2·3	4·0	3·0	1·6	1·8	1·1
	Phenethicillin	1·7	4·4	7·3	7·1	4·2	3·6	3·4
	Penicillin V	0·8	2·5	3·5	4·2	3·2	2·2	1·7
	Penicillin G	7·0	25·9	29·4	17·6	13·1	8·6	3·3
	Carbenicillin	4·5	25·5	32·5	14·7	6·9	5·8	—
	Ampicillin	4·4	9·7	10·6	8·9	8·7	5·6	—
	Chloramphenicol	1·8	5·1	5·3	5·1	6·0	9·3	11·1
	Sulphadiazine	1·9	2·8	7·5	13·1	12·2	9·4	3·7
Thoracic lymph	Cloxacillin	2·0	4·5	3·8	2·8	2·9	1·3	1·0
	Phenethicillin	0·25	2·3	4·8	5·8	5·0	3·3	3·0
	Penicillin V	0·5	3·4	4·4	2·9	2·2	1·5	0·8
	Penicillin G	15·5	30·6	20·8	17·8	12·3	7·4	4·55
	Carbenicillin	15·5	68·0	50·0	21·75	6·3	4·6	4·3
	Ampicillin	5·3	17·2	19·3	12·9	9·2	6·8	6·7
	Chloramphenicol	1·6	3·8	4·8	5·6	5·9	5·5	8·2
	Sulphadiazine	4·2	12·7	16·55	12·7	14·6	16·4	14·5

they concluded that " the concentration of free penicillin in plasma may be a practical and conservative estimate of the free penicillin concentration in interstitial fluid ". Chisholm, Calnan & Waterworth (1968) determined the concentrations of nitrofurantoin, gentamicin and carbenicillin in plasma, thoracic and renal lymph of dogs. The thoracic lymph concentrations of carbenicillin were found to be in equilibrium with those in plasma 0·5–2 h after intramuscular administration of 20 mg/kg of the penicillin. Peak plasma concentrations were approximately twice the peak thoracic lymph concentrations. In some initial experiments with " tissue cage fluid " these authors found that carbenicillin was still present in the fluid even after the penicillin could not be detected in the plasma. In a preliminary communication (Brown, 1964) we reported on the concentrations of penicillin V, phenethicillin, cloxacillin and ampicillin appearing in the thoracic lymph and whole blood of rats following intramuscular administration of 100 mg/kg. The lymph concentrations were found to be higher than the whole blood concentrations ; however, recalculation to give the plasma concentrations, allowing for the packed cell volume of the blood, showed the concentrations in the thoracic lymph to be lower than those in plasma apart from ampicillin, for which the thoracic lymph and plasma concentrations were approximately equal. The results of our experiments in which single intramuscular injections of the antibacterial were administered to dogs are in good agreement with those of Chisholm *et al.* (1968) and Verwey & Williams (1962a, b) and indicate that penicillins, even those which are highly bound to plasma proteins, penetrate well into extravascular spaces. This observation is supported by other workers (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941 ; Brown, 1964 ; Florey, Turton & Duthie, 1946 ; Jawetz, 1946 ; McCune, 1960 ; Nathanson & Liebhold, 1946 ; Ungar, 1950 ; Weinstein, Daikos & Perrin, 1951 ; Werner, Knight, McDermott, Adams & Dubois, 1954 ; White, Lee & Alverson, 1946).

Our experiments therefore indicate that penicillins penetrate readily from plasma to extravascular fluids as represented by peripheral lymph, thereby achieving good antibacterial concentrations of the free active penicillins in the tissues. The extent of binding does not appear to influence the transfer from plasma to interstitial fluid and equilibrium between free concentration in plasma and free concentration in peripheral lymph is achieved. The two non-penicillin antibacterials, sulphadiazine and chloramphenicol, do not pass so readily from plasma to extravascular fluids, possibly indicating a less readily dissociable protein/antibacterial combination.

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(*Received November* 20, 1969)

PRELIMINARY REPORT ON EXCRETION OF PENICILLINS INTO THE URINE AND BILE

TO Mr. D. M. Brown FROM B. F. Clark DATE 7.10.63. REF.

EXCRETION OF PENICILLINS INTO URINE AND BILE

The elimination of these compounds by excretion into urine and bile is an important factor to be considered in evaluating their therapeutic use. Consequently, the urinary and biliary excretion patterns of several 6-aminopenicillanic derivatives have been investigated and preliminary studies of some of the factors which influence the excretion patterns have also been carried out.

Materials and Methods

In all experiments, the penicillins were administered as their sodium salts, all doses being given in terms of the pure compound. The antibiotics were assayed by the cup plate technique using sarcina lutea as the test organism. The zone diameters obtained for the control dilutions of antibiotics were plotted against the logarithm of the concentration; and from the regression line obtained, the concentrations of the antibiotics in the specimens were estimated by interpolation. The appropriate dilutions of the controls and samples were made in phosphate buffer pH 7.0 (M/20) except in experiments where blood concentrations were determined, in which case, controls were prepared in blood.

Urinary Excretion in Rats

100 mg/kg doses of the penicillins were administered either subcutaneously or orally to groups of 6 male albino rats deprived of food overnight of weight range 300-400 g. Urine formation was promoted by oral administration of 2 mls warm tap water for each 100 g weight of rat.

The animals were housed in pairs in metabolism cages supported over glass urine-collecting funnels and allowed drinking water ad lib. Urine was collected in sterile tubes cooled in iced water.

Anaesthesia

In some experiments conscious animals were compared with anaesthetised rats which had received intraperitoneal injections of 60 mg/kg pentabarbitone sodium at the beginning of the test.

Stress

Urine was collected from rats injected intramuscularly with 100 mg/kg ampicillin and subjected to various forms of stress:

(i) Cannulation of bile duct and restraint in close fitting wire cages,

(ii) cannulation without restraint, and

(iii) isolation in metabolism cages.

Biliary Excretion in Rats

Groups of 6 rats deprived of food overnight (wt. 300-400 g.) were anaesthetised with ether. After laparotomy, the bile duct was cannulated with polythene tubing (0.4 mm internal diameter). On recovery from anaesthesia, 100 mg/kg doses of penicillin were administered orally or intramuscularly. The bile was collected at intervals in sterile tubes cooled in iced water.

Influence of Anaesthesia and Weight in Blood Concentrations

Two groups of 6 male rats deprived of food overnight (wt. 300-400 g) were injected intraperitoneally with 60 mg/kg pentabarbitone in 0.5 ml normal saline. Each animal then received 100 mg/kg methicillin intramuscularly. 0.1 ml blood samples were taken from the tail vein at intervals up to 6 hours and mixed with 0.1 ml normal saline containing 100 units heparin.

Sixteen conscious rats of weight range 210-360 g received 100 mg/kg methicillin intramuscularly. Blood samples were taken at intervals up to 6 hours.

RESULTS

Urinary Excretion in Rats

Oral Administration - Table 1. 20-25% of cloxacillin and Penicillin V were recovered in the urine within 24 hours. Only 4% of ampicillin was recovered within this period.

Effect of Anaesthesia:

The influence of anaesthesia on urinary excretion is demonstrated by the effect of pentabarbitone sodium on the excretion of intramuscular methicillin. (Table 2). Over 6 hours the mean % recovery for conscious rats was 51.9%, and from anaesthetised rats, 69.4%. The difference was not statistically significant. During the first hour of the experiment, no urine was voided by the anaesthetised animals.

Effect of Stress:

The recovery of ampicillin in the urine of rats under stress is shown in Table 3. Close restraint and cannulation caused a reduction in the amount of ampicillin recovered. Isolation in metabolism cages reduced the urinary recovery to a lesser extent. Paired animals gave the greatest output of penicillin.

Biliary Excretion in Rats

The percentage recovery of penicillins in the bile following oral administration is shown in Table 4.

Influence of Anaesthesia and Weight on Blood Concentrations

Pentabarbitone anaesthesia depressed the blood concentrations of methicillin reached after intramuscular administration of 100 mg/kg (Figure 1). Peak concentrations which occurred during the first hour after injection, were reduced by about 40%. Subsequently the concentration in the blood of the anaesthetised rats fell at a slower rate than in the conscious animals.

Peak blood concentration of methicillin varied with the weight of the rat injected. Large rats of about 300 g gave peaks which were approximately double that of animals at the lower end of the weight range, (Figure 2).

DISCUSSION

Oral absorption of penicillins in the rat is poor. About 30% of Penicillin V and cloxacillin is recovered in the urine (20%) and bile (10%) while only about 4% of ampicillin is recovered in the urine and 4% in the bile. The bile would therefore appear to be an important route of elimination in the rat.

Anaesthesia depresses the blood concentration but the overall quantity of antibiotic excreted in the urine is unaffected in spite of an initial lag period where little or no urine is formed as a result of the anaesthetic.

Other factors influencing blood concentration are:-

(i) weight, where the heavier the animal results in higher blood concentrations,

(ii) stress, where the influence of an operative procedure or a psychological stress in having the animals alone results in significantly lower blood concentrations.

TABLE 1

Excretion of Penicillins into Rat Urine

100 mg/kg doses of penicillins administered orally to rats. Urine collected over the following 24 hours. Results expressed as mean % of dose recovered from urine of 12 animals.

Penicillin	% of dose recovered in urine (24 hours)
Penicillin V	20.0
Ampicillin	4.0
Cloxacillin	20.0

TABLE 2

Recovery of Methicillin in Urine of Conscious and Pentabarbitone Anaesthetised Rats

Mean % recovery of methicillin in urine of groups of 10 rats over 6 hours after intramuscular administration of 100 mg/kg

	Mean Rat Weight (g)	Recovery at Hours: 0-½	½-1	1-½	2-4	4-6	Total
CONSCIOUS	304	7.7	11.3	10.3	17.0	5.6	51.9
ANAESTHETISED	305	-	-	18.8	45.0	5.6	69.4

TABLE 3

Percentage recovery of 100 mg/kg intramuscular ampicillin in urine of rats subjected to stressing experimental conditions

Treatment	No. of Rats	No. of Rats per cage	6 hrs Mean % Recovery
Restrained and bile duct cannulated	4	single	31.3
Free and bile duct cannulated	12	single	44.3
Free	12	single	64.0
Free	12	paired	86.0

TABLE 4

Mean percentage recovery of penicillins in bile over 6 hours following oral administration of 100 mg/kg to groups of 6 rats

Penicillin	% Recovery 6 hrs
Penicillin V	8.4
Ampicillin	3.3
Cloxacillin	10.0

FIGURE 1

BLOOD CONCENTRATIONS OF METHICILLIN IN CONSCIOUS AND ANAESTHETISED RATS

100 mg/kg. METHICILLIN INJECTED INTRAMUSCULARLY TO GROUPS OF 10 RATS. CONCENTRATIONS IN BLOOD DETERMINED OVER 6 HOURS AFTER INJECTION.

VERTICAL LINES REPRESENT STANDARD ERRORS OF MEANS.

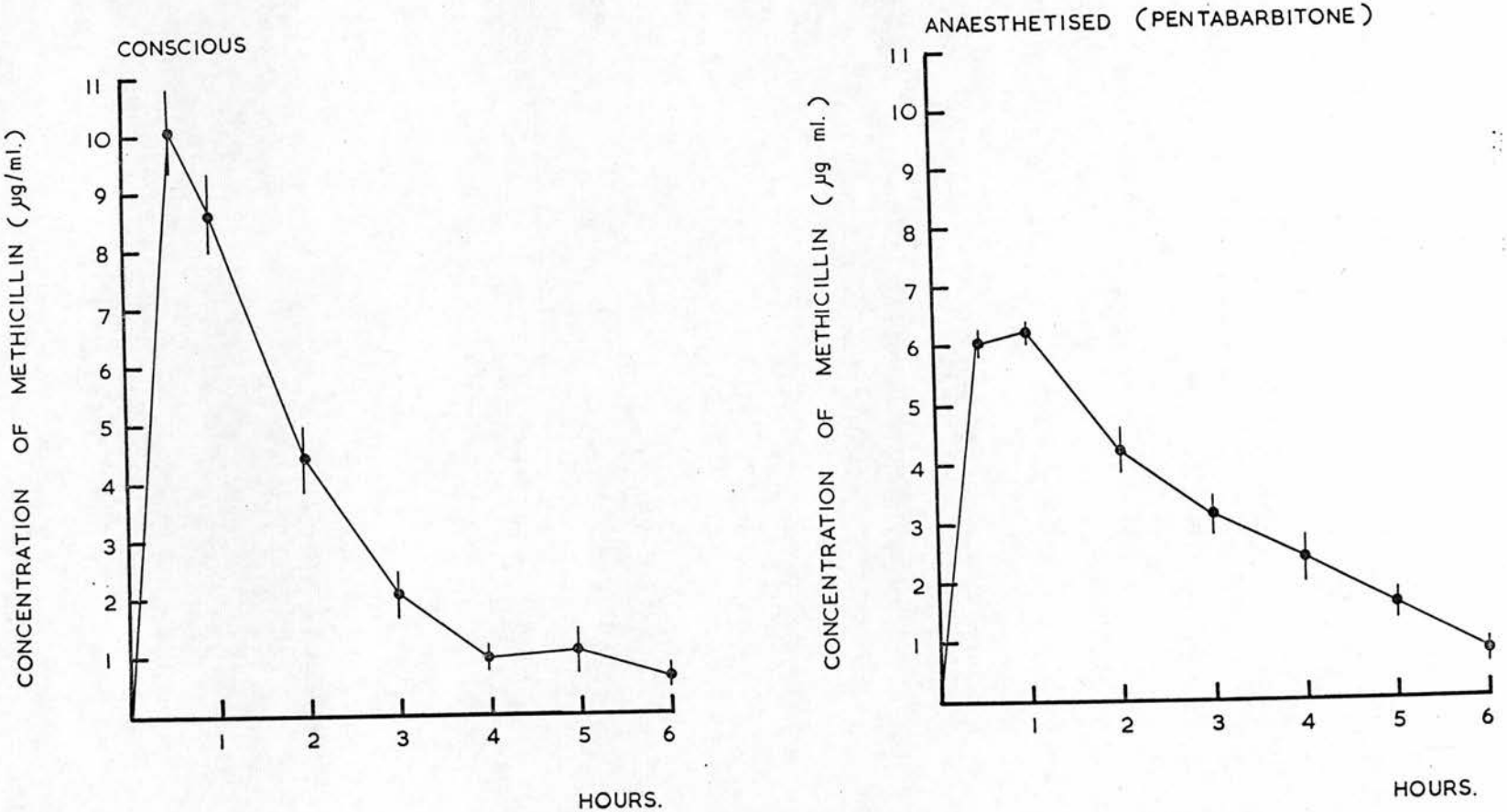
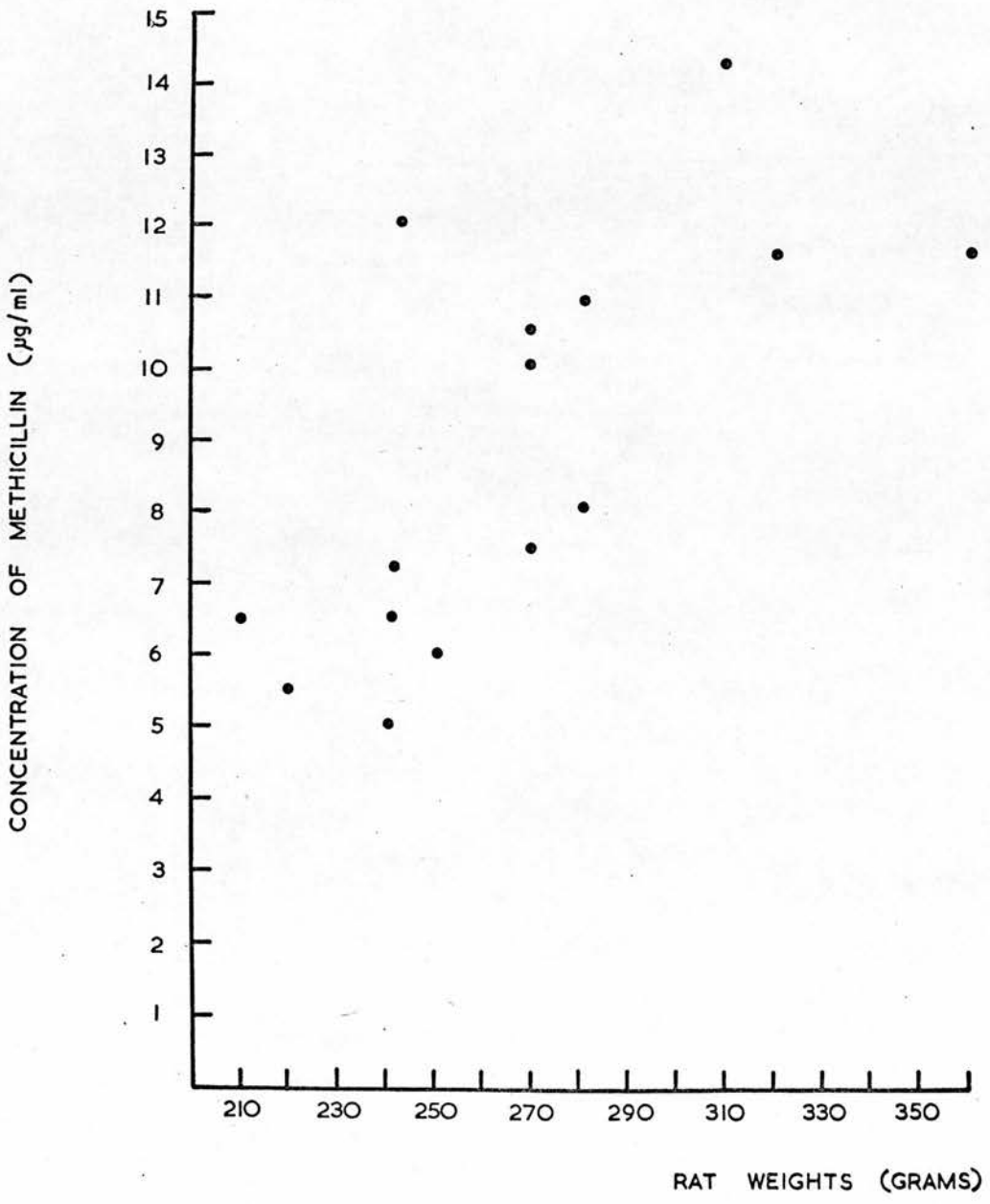


FIGURE 2

INFLUENCE OF RAT WEIGHT ON PEAK BLOOD CONCENTRATIONS OF METHICILLIN.

100 mg/kg. METHICILLIN INJECTED INTRAMUSCULARLY.

BLOOD CONCENTRATIONS DETERMINED ½ HR. AFTER ADMINISTRATION.



25. *Teratogenicity of Diphenylhydantoin in Swiss-Webster and A/Jax Mice.* J. E. GIBSON and B. A. BECKER, Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, Iowa.

Diphenylhydantoin increases incidence of cleft palate in A/Jax mice, which are genetically prone to that malformation. The purpose of this study is to evaluate diphenylhydantoin-induced teratology including cleft palate in a nonprone mouse strain. Diphenylhydantoin, 50 mg/kg, sc, was administered to primigravid Swiss-Webster and A/Jax mice during early (days 7, 8, 9) and late (11, 12, 13) embryogenesis. Litters were delivered by cesarean section on gestational day 18, weighed, examined, and measured. Resorption rates were not altered in either strain treated during early embryogenesis. A/Jax, but not Swiss-Webster, mice treated during late embryogenesis had a significant ($P < 0.05$) increase in resorptions in treated *vs* A/Jax control mice. Diphenylhydantoin resulted in reduced fetal weight in both strains when given during late embryogenesis. Fetal long-bone length, measured in alizarin red-stained preparations was shortened only in late-treated Swiss-Webster mice. The incidence of cleft palate, corrected for cleft palate occurring with cleft lip, was significantly elevated in late, but not early, treated progeny of both strains: Swiss-Webster (7/46) and A/Jax (8/26) *vs* 0/37 and 1/24 for the respective controls. Cleft palate with cleft lip occurred only in A/Jax mice; the rate was not different from vehicle-treated controls. No other soft tissue malformations were found in either strain following Bouin's fixation and freehand cross-section examination. In summary, diphenylhydantoin produced micromelia and cleft palate in Swiss-Webster mice, which are not genetically prone to cleft palate.

26. *Effect of Antibiotics upon Pregnancy in the Rabbit.* D. M. BROWN, K. H. HARPER, A. K. PALMER, and S. A. TYSH, Beecham Research Laboratories, Betchworth, Surrey, England, and Huntingdon Research Centre, Huntingdon, England.

Previous communications to this Society [Gray and Lewis, *Toxicol. Appl. Pharmacol.* 8, 342 (1966); Madisseo *et al.*, *ibid.* 10, 379 (1967)] dealt with the lethal and abortifacient actions of antibiotics in pregnant rabbits. Studies in our laboratories have confirmed and extended these observations with a number of antibiotics.

Tetracycline and penicillins in general proved the most toxic, therefore meaningful teratogenicity data could not be achieved with conventional tests. Chloramphenicol, polymyxin B, colomycin, and cycloserine, on the other hand, were well tolerated.

Because of the probable role of the gut flora in determining the effect of antibiotics it was suggested that the suckling rabbit, known to have an incomplete flora, might be conditioned to a particular antibiotic by gradually increasing the dosage, thus providing "antibiotic tolerant" adults for teratogenic studies.

To test this hypothesis, groups of weanling New Zealand White rabbits were treated with Pen G, Pen V, cloxacillin, dicloxacillin, methicillin, or tetracycline at an initial daily dosage of 10 mg/kg increasing weekly by 10 mg/kg until a dosage of 100 mg/kg was reached; this was maintained through mating and gestation.

Although some animals died before the dose reached 100 mg/kg, survivors were able to support pregnancy; there was no increased incidence of abortion and no evidence of a teratogenic action. There was some evidence of embryonic death related to maternal condition.

It is felt therefore that abortifacient and embryotoxic response to penicillins and tetracycline relates to the deterioration in maternal condition rather than to direct action on the embryo.

27. *Development of Mouse Populations for Use in Toxicology.* E. D. PALMES and J. DELPUP, Institute of Environmental Medicine, New York University Medical Center, New York, New York.

An attempt was made to develop mouse populations which could be used as test objects in toxicity studies. It was possible to breed and maintain four populations of 400 mice each living at a population density of about 1 mouse per 4 square inches. Females are removed from

PHARMACOLOGICAL PROPERTIES OF ESTERS OF 1-ALKYL-2-HYDROXYALKYLPYRROLIDINE AND THEIR QUATERNARY DERIVATIVES

BY

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BY

P. ACRED, E. M. ATKINS, J. G. BAINBRIDGE, D. M. BROWN, R. M. QUINTON AND D. TURNER

From the Pharmacology Department, Beecham Research Laboratories Ltd., Betchworth, Surrey, and C. L. Bencard Ltd., Park Royal, London

(RECEIVED MAY 13, 1957)

A series of esters of 1-alkyl-2-hydroxyalkylpyrrolidine and their quaternary derivatives have been shown to possess significant anti-acetylcholine activity. The benzilic acid esters were the most active, followed by xanthene-9-carboxylic acid, fluorene-9-carboxylic acid and diphenylacetic acid esters in that order. The quaternary derivatives were more active than their corresponding tertiary compounds both *in vivo* and *in vitro*. The most active compound of the series tested *in vivo* was (1-methylpyrrolid-2-yl)methyl benzilate methiodide and was as potent as atropine. There was a progressive decrease in anti-acetylcholine activity and a proportional increase in local anaesthetic activity as the number of carbon atoms was increased from 1 to 3 in the pyrrolidyl side-chain of the tertiary salts of the benzilic acid ester series. Likewise increasing the size of the group on the nitrogen atom led to a decrease in anti-acetylcholine activity and an increase in local anaesthetic activity. Quaternization of the tertiary salts resulted in a loss of local anaesthetic activity. Most of the compounds tested possessed some antihistamine properties, while papaverine-like activity was confined to the tertiary salts only. No significant neuromuscular blocking activity was evident.

There have been numerous attempts to modify the structure of atropine with a view to minimizing its undesirable side effects, and many simple esters have been prepared which have shown anti-acetylcholine activity. This paper describes the pharmacology of the esters of pyrrolidyl alcohols synthesized by Doyle, Mehta, and Sach (unpublished observations). Of these compounds only the benzilic and diphenylacetic acid esters of 1-methyl-2-hydroxymethylpyrrolidine have previously been reported (Blicke and Lu, 1955).

METHODS

Tests in Vitro

Anti-acetylcholine Action. — Segments, approximately 2 cm. long, were removed from the lower ileum of young guinea-pigs weighing not more than 250 g. and suspended in oxygenated Tyrode solution at 37° in a 5 ml. bath. Acetylcholine (ACh), which was allowed to act for 30 sec., was added at 2 min. intervals until constant responses were obtained. The doses of atropine and test compound were added 30 sec. before ACh and were adjusted so that the ACh response was reduced to approximately 30% and 70% of the original. Between the successive doses of antagonist the ACh response was allowed to return to normal. A 16-point assay was then performed using a Latin square design (Fisher and Yates, 1953) and the results analysed by the procedure of Schild (1942).

Segments 2 to 3 cm. long, cut from the mid-portion of the jejunum of young rabbits weighing approximately 1.5 kg., were suspended in Tyrode solution at 37°. An identical statistical procedure to that used for the guinea-pig ileum anti-ACh assay was employed. A 4 min. cycle was used. The antagonist was added 60 sec. before the ACh. Fresh rabbit jejunum often gave irregular responses to ACh for the first 1 or 2 hr.; regular responses were obtained more rapidly, without loss in sensitivity or accuracy, with gut which had been stored for 18 hr. at 7°.

Antihistamine Action.—Preparations of guinea-pig ileum were made as for the anti-acetylcholine test. Histamine was added at 90 sec. intervals until the responses were regular. The antagonist was then added to the bath 30 sec. before the histamine and the depression in responses compared with that obtained by a standard antihistamine drug. If the activity of the unknown exceeded that of the standard, a 16-point Latin square design assay was performed.

Papaverine-like Activity.—A strip of rabbit jejunum 2 to 3 cm. long was suspended in Tyrode solu-

tion at 37° in a 5 ml. bath. Spasm was induced with barium chloride (250 μg./ml.) and the spasmolytic added 2 min. later, while the barium chloride was still present. The compound was allowed to act for a further 2 min. and then washed out. The degree of relaxation of the barium spasm produced by the compound under test was compared with that produced by papaverine HCl. Two further washings were performed at 5 min. intervals during the 15 min. cycle employed.

Curare-like Activity.—The rat phrenic nerve-diaphragm preparation, as described by Bülbring (1946), was used.

Langendorff Heart.—The heart from a guinea-pig weighing approximately 400 g. was removed under ether anaesthesia and connected by an aortic cannula to a perfusion system. Oxygenated Locke solution containing $NaHCO_3$ 0.5 g./l. was allowed to flow through a warming coil to the heart. The perfusion pressure was maintained between 60 and 70 cm. water. Drugs were injected in 0.05 to 0.1 ml. Locke solution close to the heart. The amplitude and frequency were recorded and the rate of coronary outflow was measured by a Gaddum drop recorder.

Tests in Vivo

Acute Toxicity.—Acute intravenous toxicities were estimated in male albino mice (18 to 22 g.); 10 mice were used with each dose and the results were analysed according to the method described by Finney (1952).

Anti-acetylcholine Action on Cat Blood Pressure.—The method of Kühl (1925) was used. Cats were anaesthetized with ether followed by chloralose-urethane mixture (40 mg./kg. chloralose, 160 mg./kg. urethane intravenously). Blood pressure recordings were made from the carotid artery and injections were made through a polythene cannula inserted into the femoral vein. The compounds were dissolved in saline.

A dose of ACh (0.5 to 1.0 μg.), which gave a just submaximal depression in blood pressure, was administered intravenously at 2 min. intervals until a regular depression of the blood pressure was obtained. The % depression of the ACh response caused by a small intravenous dose of spasmolytic given 1 min. previously was measured and matched with that caused by a dose of atropine (0.2 to 5.0 μg.). Small doses were employed so that recovery was rapid and several compounds could be assayed on each animal. Each drug was tested on several animals and the results averaged.

Intestinal Motility.—Decerebrate cats were used. A midline stab incision was made in the abdomen. The intestine was drawn out through the incision and glass cannulae were inserted into the duodenum and jejunum or ileum. The cannulae were bent at right angles so that when in position the longer arm was vertical. A ligature was tied round the intestine 5 to 10 cm. from the cannula and the loop was filled with liquid paraffin to give an internal pressure of about 10 cm. water. The cannula was connected to an air tambour and the intestine replaced in the abdominal cavity. Handling of the intestine was reduced to a minimum. After 2 to 3 hr., when the effects of the anaesthetic had worn off, kymographic recordings of intestinal movements were made. Compounds were injected intravenously in saline through a cannula inserted in the femoral vein.

Anti-acetylcholine Activity on Guinea-pig Colon.—Guinea-pigs were anaesthetized with urethane (2 g./kg. subcutaneously), and a cannula was tied into the colon. Carbachol (1 to 2 μg./guinea-pig) was injected intravenously, at 3 to 5 min. intervals, through a cannula in the jugular vein until the intestine responded regularly by contraction. A single dose of compound was administered intravenously and two further injections of carbachol were made. If there was no response to these, the drug was considered to be active at the dose given. From 2 to 6 animals were used at each dose level. The activity was expressed in terms of minimal effective dose.

Mydriatic Activity.—Assays for mydriatic activity were carried out on the mouse according to the method of Pulewka as used by Ing, Dawes, and Wajda (1945). The pupil diameter was measured in arbitrary units with the aid of a micrometer scale set in the eyepiece of a dissecting microscope (×10). Compounds were given subcutaneously or orally to groups of 5 mice (18 to 22 g.). The pupil diameter was measured at 5 min. intervals over a period of 30 min. Atropine was used as a standard. The results were assessed at the time of maximum activity, which was usually 20 min. after administration.

Local Anaesthetic Activity.—The intradermal weal method described by Bülbring and Wajda (1945) and modified by Somers and Edge (1947) was used to determine local anaesthetic activity. Guinea-pigs weighing 400 to 450 g. were employed.

A modification of the corneal reflex method described by Chance and Lobstein (1944) was also used. Three dose levels of both test and standard compound were employed and 2 guinea-pigs were used for each dose. The solutions of the compounds were made in phosphate buffer at *p*H 7.3. The standard (procaine HCl) solution was applied to one eye and the test solution to the other. The cornea was then touched lightly with a thin bristle 6 times every 2 min. for 20 min., and the number of times that the animal failed to blink was noted. After a 4 hr. period, the same animals were used again in a crossover test. The results, expressed as % response, were plotted against log concentration and analysed according to the method described by Burn, Finney, and Goodwin (1950).

Salivary Flow.—The effect of compounds on salivary flow was estimated by the method employed by Issekutz (1917), as modified by Brown and Quinton (1957).

Gastric Secretion.—The method used was essentially that of Shay, Komarov, and Fels (1945). Six male albino rats weighing 100 to 200 g. were starved for 18 to 24 hr. in cages with wide-mesh wire bottoms to

prevent coprophagy. The duodenum was then ligated under ether anaesthesia and 4 ml. of warm saline was given intraperitoneally. Three rats were given 5 mg./kg. of the test compound subcutaneously, while the remaining three were given 1.0 ml. saline. The animals were returned to their cages and killed 6 hr. later. The oesophagus was ligated and the stomach was removed and cleared of adhering mesentery, dried with blotting paper, and the contents drained through a slit into the centrifuge tube. The gastric contents were centrifuged for 10 min. at approximately 4,000 rev./min. and the total volume and the volume of solid contents measured. 1 ml. of the supernatant was titrated with N/100 NaOH for free and total acid, Topfer's reagent being used as indicator.

Neuromuscular Blocking Action.—The decerebrate cat gastrocnemius-sciatic nerve preparation was used. Stimuli of 2 to 3 V., frequency 7/min., were applied to the sciatic nerve. Compounds at 1/20th and 1/10th of their respective intravenous LD50 values were administered in saline at 10 min. intervals through a cannula in the femoral vein.

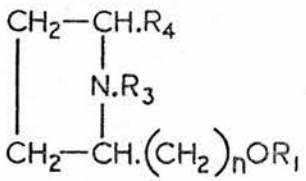
Results

Anti-acetylcholine Activity on Isolated Guinea-pig Ileum and Rabbit Jejunum.—The result of the anti-ACh activity of each compound on the isolated guinea-pig ileum (Table I) represents the weighted mean of at least four separate assays. The standard error of the combined mean values was calculated, giving on average a standard error of the order of 5% ($P=0.05$). At least two anti-ACh assays were also performed on the rabbit jejunum, and, as a rule, the relative potencies were less, generally by a factor of about 25%, than

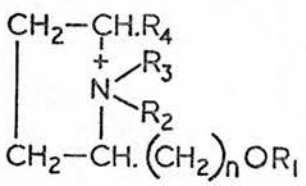
TABLE I

INTRAVENOUS TOXICITIES, ANTI-ACETYLCHOLINE AND SPASMOLYTIC ACTIVITIES OF A SERIES OF TERTIARY AND QUATERNARY SALTS OF PYRROLIDYL ALCOHOLS ESTERIFIED WITH VARIOUS ACIDS. ACID RADICAL R_1 DERIVED FROM DIPHENYLACETIC ACID (GROUP A); BENZILIC ACID (GROUP B); XANTHENE-9-CARBOXYLIC ACID (GROUP C); FLUORENE-9-CARBOXYLIC ACID (GROUP D)

The asterisk indicates an ester of the secondary base.



Tertiary esters



Quaternary esters

Ester	BRL No.	n	R_2	R_3	R_4	LD50 (intraven.) mg./kg.	Anti-ACh: Isol. G-Pig Ileum	Anti-ACh: Cat B.P.	Mydriasis (Atropine = 1·0)	Saliv. Flow	Anti-Hist. (Antazoline SO_4 = 1·0)	Anti-$BaCl_2$ Spasm. (Papaverine HCl = 1·0)
						Tertiary Esters						
A	373	2	—	CH_3	H	26·0	0·58	0·01	—	—	0·08	1·0
A	432	2	—	C_2H_5	H	15·0	0·22	0·01	—	—	0·04	1·0
B	485	1	—	CH_3	H	32·5	0·65	0·10	0·35	0·02	1·0	1·0
*B	539	2	—	H	H	27·0	0·61	0·01	<0·05	0·04	0·02	0·4
B	416	2	—	CH_3	H	28·5	0·99	0·10	0·19	0·06	0·13	0·75
B	433	2	—	C_2H_5	H	15·4	0·64	0·1	0·13	0·05	0·02	0·8
B	529	2	—	C_3H_7	H	10·5	0·18	0	<0·05	0·01	1·0	2·0
B	447	2	—	CH_3	CH_3	15·5	0·36	0·01	<0·05	0·01	0·15	1·0
B	490	3	—	CH_3	H	11·5	0·12	0	0·1	0	0·01	1·0
C	448	2	—	CH_3	H	24·5	0·93	0·01	—	—	0·15	1·0
D	417	2	—	CH_3	H	21·5	0·48	0·01	—	—	0·20	1·5
						Quaternary Esters						
A	424	2	CH_3	CH_3	H	3·0	0·45	0·03	—	—	0·1	0
B	499	1	CH_3	CH_3	H	21·5	1·02	1·0	1·09	2·03	0·08	0
B	526	1	CH_3	C_2H_5	H	14·0	1·29	0·5	1·03	0·42	0·06	0
B	425	2	CH_3	CH_3	H	22·0	2·02	0·15	0·17	0·69	0·1	0
B	481	2	CH_3	C_2H_5	H	13·0	2·72	0·40	1·33	0·95	0·15	0
B	494	2	C_2H_5	C_2H_5	H	10·0	0·23	0·06	0·25	0·06	0·03	0
B	521	2	CH_3	C_3H_7	H	8·9	0·24	0·12	0·28	0·19	0·01	0
B	454	2	CH_3	CH_3	CH_3	12·0	0·09	0	<0·05	0·01	0·01	0
B	564	3	CH_3	CH_3	H	5·4	0·53	0	<0·15	0·02	0	0
C	453	2	CH_3	CH_3	H	4·7	1·18	0·2	—	—	0·01	0
D	438	2	CH_3	CH_3	H	4·4	0·45	0·07	—	—	—	0
Atropine						82·0	1·00	1·0	1·0	1·0	0·03	0
Atropine methonitrate						8·8	0·87	1·5	—	1·65	0	0
Homatropine						84·0	0·26	—	—	0·01	0·01	0
Oxyphenonium						16·0	0·64	0·4	0·93	0·62	0	0
Methantheline						6·6	1·73	0·25	0·12	—	0·01	0
Propantheline						3·9	2·09	1·0	0·6	1·13	0·15	0

those obtained on the guinea-pig ileum. The most active compound tested on isolated guinea-pig ileum was β-(1-methylpyrrolid-2-yl)ethyl benzilate methiodide (BRL 481). On the other hand (1-methylpyrrolid-2-yl)methyl benzilate (BRL 485) was the most active compound of the series on the rabbit jejunum. On this tissue, however, methantheline was the most active of all the compounds tested. The quaternary derivatives were more active than their corresponding tertiary salts.

Antihistamine, Papaverine-like, and Curare-like Actions in Vitro.—Most of the compounds showed some detectable antihistamine action, compounds BRL 485 and BRL 529 having activities equal to that of antazoline sulphate. Most of the tertiary salts exhibited a papaverine-like action against barium-chloride-induced spasms of the rabbit jejunum equal to that of papaverine HCl. Compound BRL 529 was the most potent, being twice as active as papaverine. Quaternization destroyed or greatly reduced this property. Potencies less than 0.05 of papaverine HCl were taken as nil.

No significant curare-like action could be detected in any of the compounds.

Isolated Mammalian Heart.—Atropine in single doses up to 100 μg. usually had no effect on the amplitude, or was slightly stimulant; it had no effect on the rate or coronary flow of the perfused heart. In doses over 20 μg. the tertiary amine esters of benzilic acid appeared to have negative inotropic and chronotropic actions, which were particularly marked with BRL 416. A dose of 50 μg. of this compound usually produced a pronounced decrease in all cardiac functions leading to complete ventricular arrest. The secondary base BRL 539 and the ethyl-compound BRL 433 were equally depressant, whereas the short- and long-chain homologues BRL 485 and BRL 490 showed less activity in this respect. The quaternary amines had generally no action or a slight positive inotropic effect with high doses.

Toxicities.—The acute intravenous toxicities of the compounds are given in Table I. In general there was an increase in toxicity with increase in length of the side chain attached to the pyrrolidine nucleus and with the size of the group attached to the nitrogen atom. Quaternization of the tertiary compounds invariably led to greater toxicity, although the tertiary salts showed signs of possessing central stimulant properties absent from the quaternary derivatives.

BRL 485 and BRL 416 were the least toxic of the tertiary salts, while BRL 499 and BRL 425, their methyl quaternary analogues, were approximately one and a half times as toxic, but were the least toxic of the quaternary salts. On the other hand, atropine methonitrate was much more toxic than atropine.

Anti-acetylcholine Effect on Blood Pressure.—The results obtained are given in Table I. Compound BRL 499 was the most active of the compounds tested in the BRL series and was equal to propantheline in this respect. Compounds BRL 526 and BRL 481 had also considerable activity. The recovery from most of the compounds followed much the same pattern as after atropine.

Gastro-intestinal Motility.—There was no apparent relationship between the effect of drugs on the gastro-intestinal motility of the cat and their action in antagonizing acetylcholine on the blood pressure of the cat. An inhibitory response of the intestine was usually obtained from the first injection of the drug, but the response to a second injection was variable. In many experiments a second response was frequently not obtained and the gut became completely refractory to anti-ACh drugs. This made the interpretation of results and a direct comparison of activities difficult. We also found that the charcoal meal test in mice gave no clear guide to intestinal inhibition by anti-ACh drugs.

Anti-acetylcholine Action on Guinea-pig Colon.—In view of the wide variability obtained with the preceding method and the uncertainty of correct interpretation, it was decided to use carbachol as an intestinal stimulant, and to note the depressant effect of compounds on this response. Difficulties were again experienced in that the intestines of some animals were found to be completely resistant to carbachol, even though profuse salivation occurred in response to the injections. Furthermore, a response to carbachol tended to appear and disappear spontaneously during the course of the experiment. For this reason the experiments were designed to be of short duration, and all animals which failed to respond to an initial dose

TABLE II

MINIMUM EFFECTIVE INTRAVENOUS DOSE REQUIRED TO INHIBIT CARBACHOL-INDUCED CONTRACTION OF GUINEA-PIG COLON

Compound	Minimum Effective Dose (μg./kg.)
Atropine	8
Oxyphenonium ..	32
Propantheline ..	Variable 16 to 64
BRL 485	Variable
BRL 499	8
BRL 526	64
BRL 416	64
BRL 425	16
BRL 433	64
BRL 481	32
BRL 521	64

of carbachol were discarded. The results obtained with the more active compounds of the benzilic acid series (group B) are given in Table II. A typical response to carbachol and its inhibition by an anti-ACh drug (BRL 499) is seen in Fig. 1. This compound proved to be the most active synthetic compound tested.

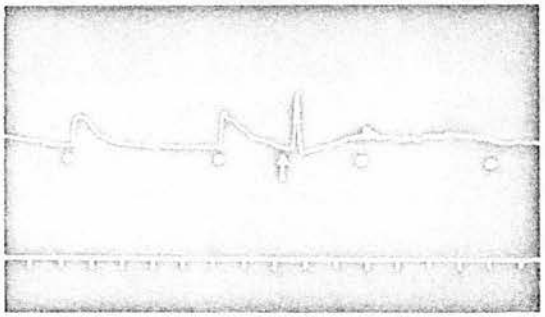


FIG. 1.—Recording of the response of the guinea-pig colon following 1 μg. carbachol administered intravenously at the dots and the inhibition produced by compound BRL 499 (16 μg. kg.) given at the arrow. Time, 1 min.

Mydriasis.—All the benzilic acid esters were tested for their mydriatic action. By the subcutaneous route compounds BRL 481, BRL 526 and BRL 499 were all of the same order of activity as atropine. Oxyphenonium and propantheline were somewhat less active and all the other members of the BRL series were much less active.

Given orally, only the tertiary salts showed regular absorption and gave repeatable results, but none was as active as atropine. The ratio of potencies of BRL 485 and BRL 416 to atropine between the subcutaneous and oral routes was, however, of the same order. The quaternary salts were absorbed erratically and required a dose of the order of 100 times the subcutaneous dose to show an effect. Even with such a dose, no significant absorption occurred in a considerable % of animals.

Local Anaesthetic Activity.—Selected compounds were tested for local anaesthetic activity, which was found in tertiary salts but not in quaternary salts. The results are given in Table III.

Salivary Flow.—The regression for inhibition of salivation by atropine against log dose was linear in two sets of 8 rabbits. Dose-response curves were also found for compounds BRL 416 and BRL 425 on the first set of rabbits and for compound BRL 499, oxyphenonium, propantheline and atropine methonitrate on the second set of rabbits. Since none of the curves obtained showed

TABLE III

COMPARISON OF LOCAL ANAESTHETIC ACTIVITY OF TERTIARY SALTS WITH PROCAINE HCl

The effect of increasing the number of carbon atoms in side chain, the size of group on the nitrogen atom, and of altering the acidic radical is recorded. See Table I for explanation of n, R_3, and R_4.

Compound	n	R_3	R_4	Corneal Reflex (Procaine HCl = 1)	Intradermal Weal (Procaine HCl = 1)
BRL 485 ..	1	CH_3	H	2·05	0·2 to 0·5
BRL 416 ..	2	CH_3	H	16·5	2·9
BRL 490 ..	3	CH_3	H	29·3	4·9
BRL 433 ..	2	C_2H_5	H	26·6	8·6
BRL 447 ..	2	CH_3	CH_3	28·6	
BRL 529 ..	2	C_3H_7	H	36·5	7·4
BRL 539 ..	2	H	H	0·2 to 0·5	
BRL 373 ..	2	CH_3	H	13·8	

significant deviation from parallelism or linear regression, it was decided to estimate activity at one dose level and to assume that the dose-response line passing through the point obtained would run parallel to the standard atropine curve. The dose which gave a 50% depression in response (ED50) was found by interpolating on the line so obtained. The relative potencies are given in Table I.

Gastric Secretion.—A quantitative assay was impractical owing to the large variation in the amount of acid secreted in untreated rats. The activities have, therefore, been measured in terms of an "all or none" response. All the compounds tested produced a significant reduction ($P=0.05$) in the volume of gastric secretion, except compounds BRL 485, BRL 447, BRL 454, BRL 481, and BRL 417.

Cat Gastrocnemius-sciatic Preparation.—None of the compounds had any significant action on the response of the gastrocnemius muscle to sciatic nerve stimulation.

DISCUSSION

All the compounds of the BRL series showed anti-ACh activity both *in vitro* and *in vivo*. On the basis of the *in vitro* comparative assays, the benzilic acid esters were the most active, followed by xanthene-9-carboxylic acid, fluorene-9-carboxylic acid and diphenylacetic acid esters, in that order.

Detailed study of the benzilic acid esters showed that maximum activity on the guinea-pig ileum occurred when the carbon side-chain attached to the pyrrolidine nucleus contained two carbon atoms. When the side-chain was kept constant, an increase in the size of the group on the nitrogen atom resulted in a proportional decrease in *in vitro* anti-ACh activity.

There was little difference between the tertiary compounds containing one or two carbon atoms in the side-chain in their antagonism of the vaso-depressor action of ACh, although the shorter chain compound appeared to be more active as a mydriatic and the two carbon chain compound more active as an anti-salivatory agent. The three carbon chain compound was, however, much less active as an anti-ACh agent. The short chain compound tended to be somewhat stimulatory and produced variable results against carbachol stimulation of the guinea-pig colon. The precise activity could not be measured, but appeared to be much less than that shown by the two carbon chain compound. The three carbon compound had little activity.

A feature of the tertiary derivatives was their local anaesthetic activity, the quaternary compounds being completely inactive in this respect. As the anti-ACh action decreased, the local anaesthetic activity increased in proportion to the number of carbon atoms in the side-chain or attached to the nitrogen. Relative to procaine hydrochloride the compounds were much more active on the corneal reflex test than by the intradermal weal test, indicating that they penetrate mucous membranes. On the corneal reflex β-(1-ethylpyrrolid-2-yl)ethyl benzilate (BRL 433), however, appeared to be more active than β-(1-*n*-propylpyrrolid-2-yl)ethyl benzilate (BRL 529), which has a larger group on the nitrogen. This discrepancy is probably due to the error of the assay, which, by the nature of the test, was large.

All the tertiary salts were active orally. This was well shown by the oral mydriatic test. In mice, absorption from the alimentary tract was poor and erratic with all quaternary derivatives. Tests in human volunteers, however, indicated that BRL 499 was regularly absorbed at a dose of 5 mg. after oral administration.

The quaternary compounds having the same groups on the nitrogen atom showed maximum anti-ACh activity *in vitro* and *in vivo* when the side-chain contained only one carbon atom. (1-methyl-2-pyrrolid-2-yl)methyl benzilate methiodide (BRL 499) was the most active of all the compounds tested *in vivo*, and in every test was as potent as atropine. When the side-chain of the quaternary compounds was kept constant and the alkyl groups on the nitrogen atom increased in size, maximum *in vitro* activity occurred with the methyl-ethyl-derivatives (BRL 481 and BRL 526). In the two carbon chain series the corresponding methyl-propyl-compound (BRL 521) was comparatively much less active and had the same order of activity as the diethyl-compound (BRL 494). *In vivo*, the relative potencies of this series on salivary flow and mydriasis were greater than those for their action against ACh on the cat blood pressure and against carbachol-induced stimulation of the guinea-pig colon. In this latter test, however, activity was maximal with the dimethyl-compound (BRL 425).

The substitution of a methyl group in the 5-position in the pyrrolidine ring greatly reduced anti-ACh activity, but did not appear to influence local anaesthetic activity. Anti-ACh activity appears to depend, therefore, on the overall size of the group or groups on the nitrogen atom, on the length of the side-chain, and on the absence of other substituents in the pyrrolidine nucleus.

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THE ASSAY OF ANTI-ACETYLCHOLINE AGENTS FOR ANTAGONISM OF PILOCARPINE-INDUCED SALIVATION IN RABBITS

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THE ASSAY OF ANTI-ACETYLCHOLINE AGENTS FOR ANTAGONISM OF PILOCARPINE-INDUCED SALIVATION IN RABBITS

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Experimental conditions affecting tests of atropine-like agents for antagonism of pilocarpine-induced salivation in rabbits have been examined. A simple method of assay of such agents is described. It gave results of fair accuracy and reproducibility, permitted a full statistical analysis, and provided an estimate of the error.

The effect of drugs on salivary flow has been examined in experimental animals by several techniques. Cushny (1920) used dogs with salivary fistulae, and Bülbring and Dawes (1945) measured the flow of saliva from the submaxillary gland of anaesthetized cats by cannulation of Wharton's duct. Issekutz (1917), who collected the saliva which dripped from the mouths of rabbits under urethane, reported that 1.5 to 2.0 mg. atropine stopped pilocarpine-induced salivation. This last method, which requires little technical skill, has been adapted by many workers, but there has been little detailed analysis of the optimal conditions. Furthermore, statistical evaluation of the results has rarely been attempted and the accuracy of the methods is therefore virtually unknown. Brown and Werner (1949), indeed, found such variability between responses of different rabbits that they regarded any quantitative assay as impracticable.

This paper describes conditions that affect the responses and the accuracy of the assay and outlines a simple method which gives results whose accuracy can be readily and fully assessed.

ANALYSIS OF FACTORS INFLUENCING SALIVARY FLOW

Use of Urethane.—Unlike several workers who used unanaesthetized animals (e.g., Fromherz, 1933; Lands, Nash, and Hooper, 1946; Chen, 1954), we could not regularly obtain a suitable amount of saliva from rabbits which had received no urethane. However, a large oral dose of urethane 1 g./kg. or more was undesirable, for the narcotic action lasted some 6 to 10 hr., and the animals lost their cough and swallowing reflexes completely (Brown and Werner, 1949; Brown, Thompson, Klahm, and Werner, 1950; Hoekstra, Tisch, Rakieten, and Dickison, 1954; Tripod, 1949). The dose of urethane is therefore critical.

The variation in the amount of saliva collected in 30 min. with the dose of urethane was determined after various doses of pilocarpine, for one set of eight rabbits (Fig. 1). The drugs were given in random order, over 4½ weeks, each animal receiving every possible combination. At all three doses of pilocarpine, the slopes of the urethane dose-response lines were very steep. Indeed, the urethane dose appeared to control the response more effectively than did the pilocarpine. The urethane probably did not affect the actual rate of salivation, but only increased the efficiency of collection.

An oral dose of 0.5 to 0.625 g./kg. of urethane was effectively sedative to the rabbits without producing loss of consciousness, and a good collection of saliva was possible. Collection was more efficient from rabbits given 1.0 g./kg. urethane, but, owing to the risk of harmful effects to the animals, 0.5 to 0.625 g./kg. was preferred.

The urethane was administered orally in 25 ml. saline to ensure adequate hydration and a good reserve of body water upon which to draw during and after the profuse salivation.

No other anaesthetic was tried in these investigations.

Dose of Pilocarpine.—Doses of pilocarpine used by previous workers in this field have ranged from 0.25 mg./kg. (Fromherz, 1933) to 100 mg./kg. subcutaneously (Graham and Lazarus, 1940). The relation between the dose of pilocarpine nitrate and the salivary response was investigated over the range of 1.25 to 5.0 mg./kg., at three

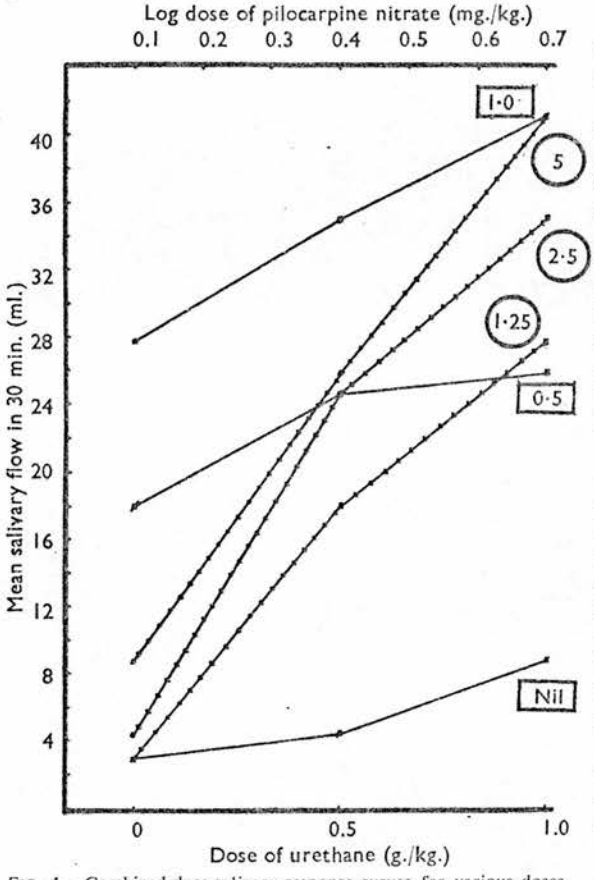

FIG. 1.—Combined dose-salivary response curves for various doses of urethane and pilocarpine, in a set of 8 rabbits. ———— Pilocarpine (log dose on top abscissa) after various doses of urethane (g./kg., in squares). ⊢—⊢—⊢—⊢—⊢ Urethane (bottom abscissa) preceding various doses of pilocarpine (mg./kg., in circles).

different doses of urethane (Fig. 1). The pilocarpine nitrate was injected subcutaneously 30 to 45 min. after the oral dose of urethane. The rabbits were then placed in their stocks and the saliva which dripped from their mouths was collected.

In the absence of urethane, an increase in the dose of pilocarpine from 1.25 to 5.0 mg./kg. had little effect on the small amount of saliva obtained. On the other hand, with 0.5 g./kg. and 1 g./kg. of urethane, the response to even 1.25 mg./kg. pilocarpine was considerably more than that to 5 mg./kg. after no urethane. When 0.5 g./kg. of urethane was given, there was little difference between the amounts of saliva secreted after the injection of 2.5 mg. and 5 mg./kg. pilocarpine. However, the higher dose was used throughout the investigations, since, after it, salivation was of convenient duration (90 min.) and of suitable intensity—not too readily antagonized by drugs, yet susceptible enough to give a sensitive assay.

Choice of Animals.—Rabbits of 1.5 kg. and over were used. Differences in sex and weight did not appear to influence the response to the drugs.

The rabbits showed enormous variability in their sensitivity to atropine. In about half of them the salivary response to pilocarpine was not affected significantly by doses of atropine up to 80 μg./kg., given subcutaneously 15 min. before the pilocarpine. On the other hand the remainder showed about a 50% decrease in flow after 5 to 10 μg./kg., and almost a 100% decrease after 25 μg./kg. This insensitivity to atropine was presumably due to the presence in the blood of an enzyme—atropinesterase (Glick, 1940)—capable of hydrolysing atropine. Serum from several resistant rabbits was able to destroy atropine *in vitro*.

Although both Glick and Glaubach (1941) and Ammon and Savelsberg (1949) have reported widely ranging figures for the effective atropinesterase activity of the blood of those rabbits possessing it, we found that rabbits showed either a large (>50%) reduction in salivary flow after 25 μg./kg. atropine, or else an insignificant reduction (<20%) after double or treble that dose. Consequently, once a consistent salivary response to the pilocarpine alone had been obtained in a rabbit, a single test with 25 μg./kg. atropine given 15 min. before the pilocarpine was sufficient for the assessment of atropine resistance. All the animals showing less than a 50% inhibition of salivation by this dose were rejected.

Interval Between Administration of Atropine and of Pilocarpine.—In order to find the optimal time interval between the injection of atropine, or similar drugs, and of pilocarpine, a random design assay was performed on one set of eight rabbits, in which the antagonism of salivary flow was measured for four different atropine-like drugs given 0, 5, 15, 35, and, on two occasions, 70 min. before the injection of pilocarpine. The drugs used were atropine sulphate, atropine methyl nitrate, benactyzine (β-diethylaminoethyl benzilate HCl) and oxyphenonium (phenylcyclohexyl glycollic acid ester of β-diethylaminoethanol methobromide). The animals received 0.5 to 0.625 g./kg. urethane orally and 5 mg./kg. pilocarpine nitrate subcutaneously.

The mean percentage inhibitions of salivation are given in Table I and the time-response curves are plotted in Fig. 2.

Atropine appeared to exhibit maximal activity when administered about 15 min. before the pilocarpine; its effect after 35 min. was less than after 5 or 15 min. Atropine methyl nitrate had a more prolonged action which reached a maximum when it was given about 35 min. before pilo-

TABLE I

THE EFFECT OF VARYING THE INTERVAL BETWEEN THE INJECTIONS OF AN ATROPINE-LIKE DRUG AND OF PILOCARPINE ON THE INHIBITION OF SALIVARY FLOW

Drug	Dose μg./kg. s.c.	Mean % Inhibition of Salivation at Different Times (min.) Between Injection of Drug and Pilocarpine				
		0	5	15	35	70
Atropine SO_4 ..	5	32·6*	45·4	55·1	36·9*	
Atropine methyl nitrate	2	24·4*	35·6*	49·9	59·3	53·5
Benactyzine	750	36·5	30·5	24·5	22·3	
Oxyphenonium ..	5	48·9*	60·9	63·9	78·5*	78·5*

S.E. of each mean, 7·2.
* Response significantly different from that at 15 min. ($P<0.05$).

carpine. Even 70 min. after administration, atropine methyl nitrate still showed a greater effect than after 0 to 15 min. Oxyphenonium, the other quaternary compound, gave identical results. With benactyzine, however, maximal activity was obtained when the drug was given at the same time as pilocarpine.

It thus appears that the times at which different atropine-like drugs exert maximal activity after subcutaneous injection vary considerably. In the assay method described subsequently, the time between administration of the atropine-like drug and the pilocarpine was 15 min., in order to standardize conditions. The activity displayed under these conditions may not be maximal, particularly with compounds containing a quaternary nitrogen atom, and the relative potencies in terms of atropine might thus be smaller than could otherwise be obtained.

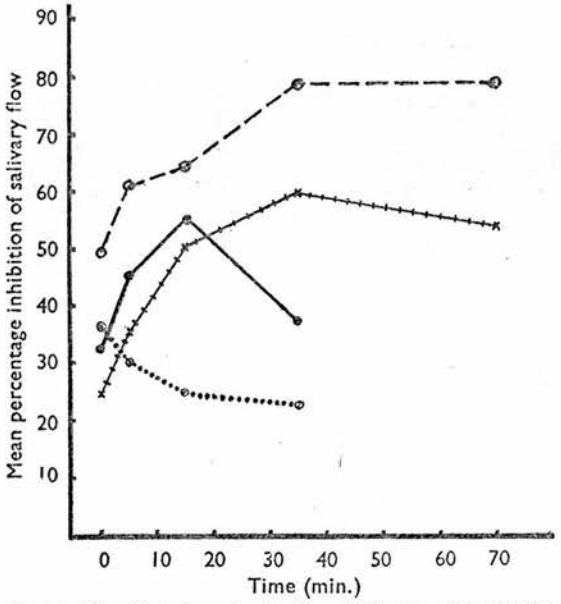

FIG. 2.—The effect of varying the time of injection of the atropine-like drug on the observed potency. Abscissa, time (min.) between injections of the drug and of pilocarpine (5 mg./kg.). Ordinate, mean percentage inhibition of salivary flow, for 8 rabbits. All drugs injected subcutaneously. ●————● Atropine sulphate 5 μg./kg. ×—|—|—|—× Atropine methyl nitrate 2 μg./kg. ●·····● Benactyzine HCl 750 μg./kg. O — — — O Oxyphenonium bromide 5 μg./kg.

ANALYSIS OF RESULTS

Regular gradation of response with dose was shown in atropine-sensitive rabbits—that is, a regular decrease of salivary flow was obtained with increasing doses of atropine. The response of each animal was expressed as the percentage depression of the total volume of saliva collected after a dose of atropine or similar agent, compared with the mean of the volumes obtained from the same animal in two control experiments. The results obtained from each of eight animals in a set were averaged. In practice, control experiments were performed only once in every six to twelve experiments, the responses in all those tests falling between any two controls being expressed with reference to the mean of these two control values. The average standard deviation of such a mean control value, calculated from figures for each of eight rabbits tested over nine months, was 10.9 ml. Collection of saliva was maintained for 90 min. after the rabbits had received pilocarpine and had been placed in their stocks. By the end of this time, the flow had ceased in all except a few control observations where it had fallen to a rate of below 0.2 ml./min.

Eight animals for each experiment gave results of adequate accuracy. A set of animals could be used twice weekly, with only very occasional rests, for at least 15 months without any ill-effects or tolerance.

A large number of drugs could therefore be compared for their activity in antagonizing pilocarpine-induced salivation, on the same set of eight rabbits.

The time course of the salivation obtained from a set of eight rabbits in control experiments and in tests with various doses of atropine is given in Fig. 3. Where no atropine was given, the salivation response to pilocarpine appeared to fall away exponentially with time. If moderate to strong doses of atropine were given 15 min. before the pilocarpine, however, the time-log response lines appeared to curve. This might indicate that atropine was less readily eliminated or removed from the receptors than pilocarpine.

The dose-response relation for atropine was investigated several times, on two different sets of rabbits. Good approximations to a linear log dose-response line were obtained over the 20 to

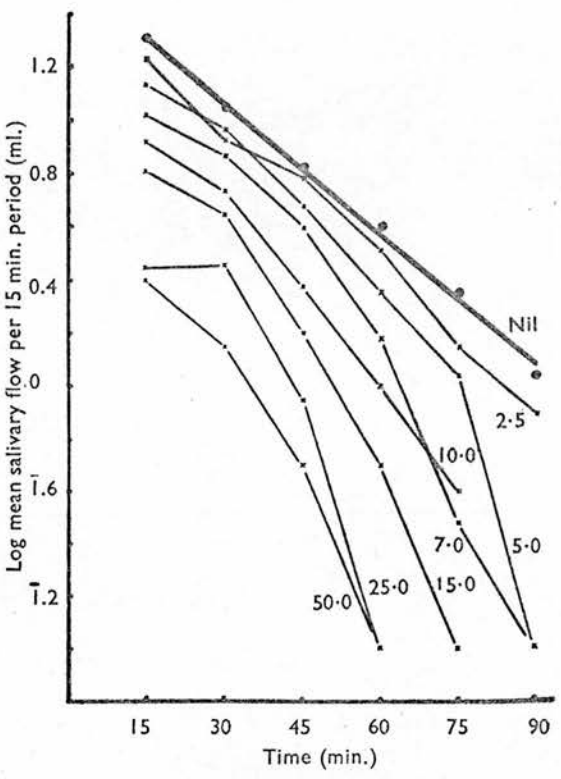

FIG. 3.—Time-salivary flow curves for a set of 8 rabbits, after 5 mg./kg. pilocarpine nitrate, preceded by various doses of atropine. Dose of atropine in μg./kg. is given alongside the appropriate line. Abscissa, time (min.) after injection of pilocarpine. Ordinate, mean log volume (ml.) of saliva for each rabbit, measured for 15 min. periods.

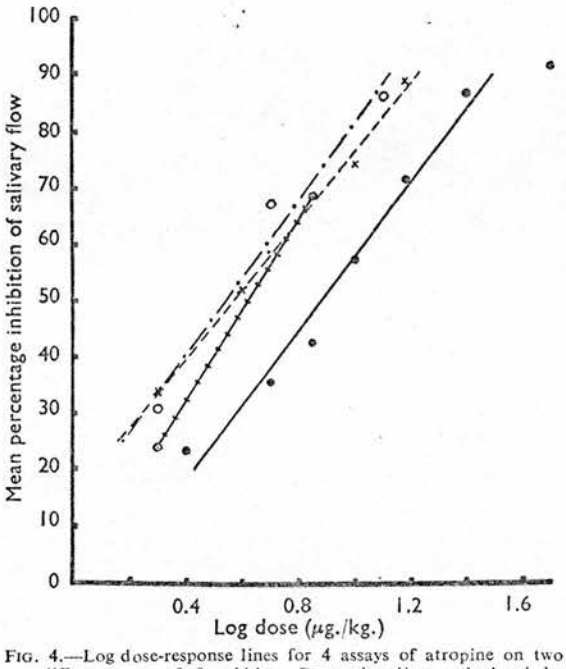

FIG. 4.—Log dose-response lines for 4 assays of atropine on two different sets of 8 rabbits. Regression lines calculated by method of least squares. ●———● Assay 5/55 on set A rabbits, × — — — × Assay 8/55, O — · — O Assay 2/56, ○—|—|—○ Assay 2/56 on set B rabbits.

90% maximal response range, and regression lines were fitted by the method of least squares (Fig. 4). The variance due to deviation from linearity was never statistically significant (i.e., $P<0.05$). Results were analysed by the standard analysis of variance method; one set of results is given in Table II. Although atropine sulphate was used, the doses were always calculated in terms of the base.

Table III gives data from five different assays, four on one set of rabbits and one on another. There is a close similarity of the results in Set B. The difference in the estimates of ED50 for atropine between the two sets of rabbits is largely an indication of differences encountered in the sensitivity of individual rabbits to atropine—even those apparently entirely lacking atropinesterase. The differences in response of these two sets of rabbits emphasize the need to compare anti-acetylcholine agents in the same group of rabbits. Many different compounds were so tested and

TABLE II

DOSE-INHIBITION OF SALIVATION DATA FOR ATROPINE ON ONE SET OF 8 RABBITS

Rabbit	% Decrease in Salivary Flow 15 min. after Atropine s.c.				Total
	Dose of Atropine (μg./kg.)				
	2	4	10	15	
1	−34	41	30	77	114
2	67	97	82	91	337
3	6	32	57	77	172
4	51	33	86	94	264
5	70	100	95	100	365
6	16	38	72	77	203
7	28	46	71	97	242
8	69	29	100	98	296
Total ..	273	416	593	711	1,993
Mean ..	34·1	52·0	74·1	88·9	

Slope $b=61{\cdot}1$. ED50 $=3{\cdot}6$ μg./kg.

ANALYSIS OF VARIANCE

Source of Variance	Sum of Squares	d.f.	Mean Square	F	P
Regression	13,903	1	13,903	39·2	<0·01
Deviation from linearity	65	2	32·5	10·9	>0·05
Between doses	13,968	3			
Between animals ..	12,488	7	1,784	5·0	0·01–0·1
Residual error	7,440	21	354·3		
Total	33,896	31			

$\lambda=0{\cdot}31$ where $\lambda=\sqrt{\frac{\text{Mean Square Error}}{b}}$ S.E. of $b=9{\cdot}7$

TABLE III

RESULTS OF VARIOUS ASSAYS OF ATROPINE FOR INHIBITION OF SALIVATION

Rabbits:	Set A	Set B			
Assay No.	1	1	2	3*	4*
Date of Assay	5/55	8/55	2/56	2/56	4/56
No. of dose-levels used..	6	4	3	2	2
Slope (b)	65·7	61·1	68·0	81·3	60·3
S.E.b	11·5	9·7	7·1	17·9	12·4
ED50 (μg./kg.)	7·5	3·6	3·4	4·2	5·1
Limits of error (P=0·05) of ED50	5·8–9·9	2·9–4·8	2·8–4·2	3·5–4·9	4·2–6·1
λ	0·40	0·31	0·20	0·30	0·22

* Part of a randomized design assay incorporating two other compounds.

their potency referred constantly to that of the standard (atropine), whose effective potency appeared to stay reasonably constant for at least nine months.

Results for Other Atropine-like Agents

In addition to atropine, several other anti-acetylcholine agents were tested by this method for their antagonism of induced salivation. Atropine methyl nitrate, oxyphenonium, and propantheline were tested on rabbits of Set B at about the same time as the second atropine assay (Table III); several doses were used in each assay. For all these compounds, doses were calculated in terms of the salt. The calculated regression lines are shown in Fig. 5, and the analysis of the results given in Table IV. The variance due to linear regression was highly significant ($P<0.01$) and that due to deviation from linear regression not significant ($P>0.2$).

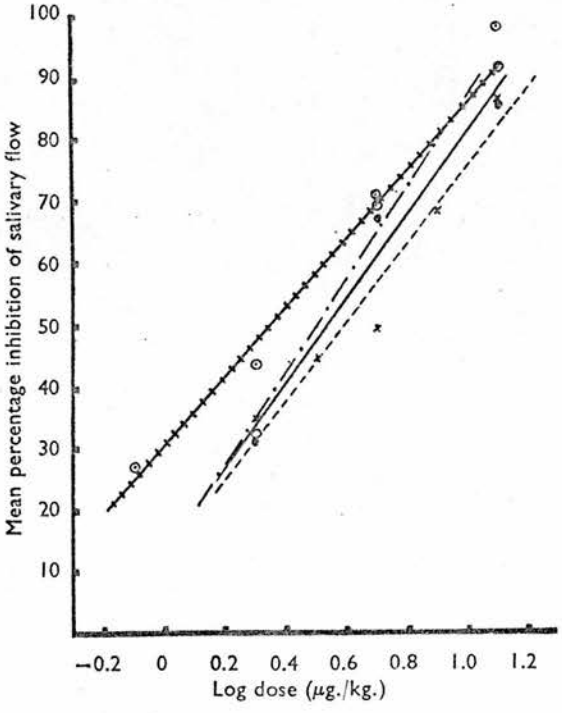

FIG. 5.—Log dose-response lines for: ●———● Atropine sulphate. ○—|—|—○ Atropine methyl nitrate. ×— — —× Oxyphenonium. O — · — O Propantheline. All results obtained on same set of 8 rabbits.

TABLE IV

ASSAYS OF ATROPINE-LIKE DRUGS FOR INHIBITION OF SALIVATION

Compound	Atropine (Sulphate)	Atropine Methyl Nitrate	Oxyphenonium	Propantheline
No. of dose-levels tested ..	3	4†	5	3
Slope (b)	68·0	55·3	63·7	74·3
S.E.b	7·1	15·2	9·4	10·2
ED50 (μg./kg.)	3·4	2·2	3·9	3·2
Limits of error of ED50 (P=0·05)	2·8–4·2	1·4–3·6	3·2–4·8	2·6–4·0
Approx. relative potency* ..	1·0	1·5	0·9	1·1
λ	0·20	0·41	0·25	0·21

* $= \frac{\text{ED50 Atropine}}{\text{ED50 Compound}}$

† Only 3 used in the analysis, the fourth giving a response over 90% of maximal.

The relative potencies of the three compounds in terms of atropine are expressed as the ratios of their ED50s (Table IV). There was little difference in the activity of these four agents, although atropine methyl nitrate appeared slightly more potent than the tertiary base.

(2+2) Assay

The assessment of the relative potency of one drug in terms of another from dose-response data obtained at different times must, however, inevitably be liable to more error than that derived from tests carried out under identical conditions for both drugs. An assay method incorporating randomized doses was therefore adopted, in which, at any one time, the four pairs in a set of eight rabbits received different doses. Two doses of each compound giving respectively about a 35% and 70% response were administered

TABLE V

COMPARISON OF ACTIVITIES OF ATROPINE-LIKE DRUGS WITH THAT OF ATROPINE

	λ	Log Relative Potency (M)	S.E. of M	Rel. Potency	Limits of Error of Rel. Potency (P=0·95)
Assay 1: Combined b=65·6 ..	0·30				
Atropine x atropine $MeNO_3$		0·357	0·104	2·28	1·40–3·70
x homatropine HBr		–2·039	0·109	0·0091	0·0055–0·0152
Assay 2: Combined b=59·8 ..	0·22				
Atropine x tricyclamol Cl		–0·211	0·086	0·62	0·41–0·93

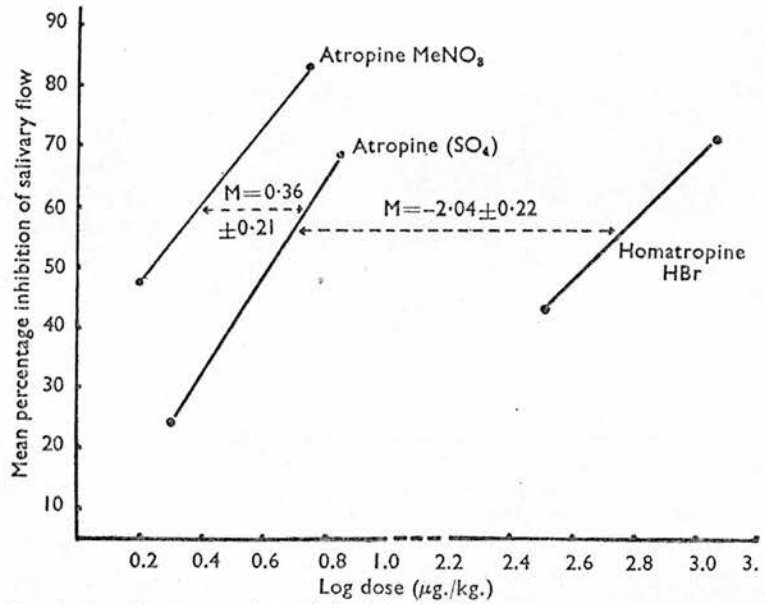

FIG. 6.—Log dose-response lines obtained for atropine sulphate, atropine methyl nitrate and homatropine HBr in a 2 + 2 + 2 assay on a set of 8 rabbits. Values for M (log relative potency) ± its limits of error ($P=0{\cdot}95$) are given, with atropine taken as standard.

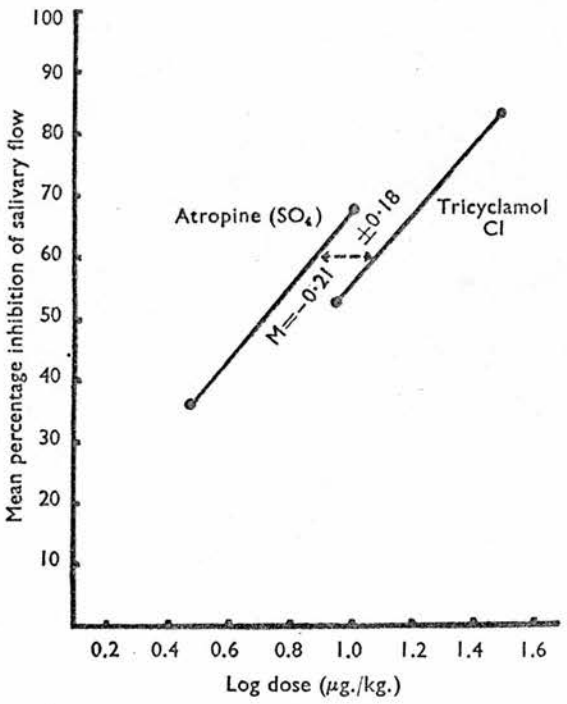

FIG. 7.—Log dose-response lines obtained for atropine sulphate and tricyclamol Cl in a 2 + 2 assay on a set of 8 rabbits. Log relative potency (M) ± its limits of error ($P=0.95$) are given, with atropine taken as standard.

subcutaneously. The ratio of the two doses was the same for all compounds in an assay. In the first assay, three compounds were tested—atropine sulphate, atropine methyl nitrate, and homatropine HBr. The doses were given in random order over six experiments. The assay results were compared with the mean result of two control experiments performed just before and just after the assay. In a second assay, tricyclamol chloride was tested against atropine in a similar manner. Analysis of results followed the same lines as before, with adaptations of the Schild (1942) and Holton (1948) methods of evaluating 2+2 assays.

The dose-response lines obtained in these assays can be seen in Figs. 6 and 7, and the relevant data tabulated in Table V. Variance due to regression was always highly significant ($P<0.01$), whereas that due to deviation from parallelism was not ($P>0.2$).

The relative potencies of atropine methyl nitrate, homatropine HBr, and tricyclamol in terms of atropine were 2.28, 0.0091, and 0.62 respectively; the difference in activity between the test compound and atropine was statistically significant with all the drugs.

Discussion

Rabbits fully sensitive to atropine are essential for a reliable assay, since the dose of atropine producing 50% inhibition of salivation in about half the animals tested was more than ten times that necessary in the other half. Serum from the former rabbits destroyed atropine *in vitro*, whereas serum from the atropine-sensitive rabbits did not. Other atropine-like drugs are also susceptible to destruction in the serum of certain rabbits; homatropine, for example, is almost as readily attacked as atropine (Ammon and Savelsberg, 1949). On the other hand, Trasentin (Glick, 1942) and Trasentin 6H and Lachesine (Blaschko, Chou, and Wajda, 1947) have been reported to be unaffected by atropinesterase, and it would be possible, therefore, to compare these compounds on atropine-resistant rabbits. Other more recently synthesized atropine-like compounds

do not appear to have been investigated for their susceptibility to destruction by atropinesterase.

The incidence and transmission of atropine resistance in rabbits have been investigated by Sawin and Glick (1943) and others; it seems to be at least partly controlled by hereditary factors. Consequently, it would be expected that for any one strain the atropine-resistance factor would be present to a fairly consistent degree, but that there would be a variation between different strains. About 90% of our "English" rabbits, bred locally, were atropine-sensitive, whereas roughly a similar percentage of various blue "Beveren" and black rabbits obtained from another dealer were resistant. According to Sawin and Glick, there may be a relation between the presence of atropinesterase in the blood and the extent of black pigment in the rabbit's coat.

Several authors testing anti-acetylcholine agents for salivary blockade in rabbits have reported a wide variability in sensitivity to atropine, but none has related this to the presence of an enzymic mechanism for destroying atropine. Brown and Werner (1949), for instance, reported that 0.075 to 0.3 mg./kg. atropine intramuscularly "markedly inhibited salivary secretion in some animals, but had little effect in others." They therefore used a quantal response, and reported that the minimal dose of atropine which would reduce the salivary flow to below 20 ml. in 2 hr. in five out of six rabbits tested was 600 μg./kg. At least some of the animals used by these workers probably possessed atropinesterase in their blood.

Cahen and Tvede (1952), in a quantal assay of salivary blockade in rabbits, showed that the percentage (probit) of rabbits with at least a 50% inhibition of pilocarpine-induced salivation was linearly related to the log dose of atropine. The ED50 for atropine by this method was 62 μg./kg., whereas assessment by graded response in the same 40 animals gave an ED50 of 110 μg./kg. Such a difference between ED50 values might be expected if it is assumed that certain of these 40 rabbits (but less than half of the total) possessed atropinesterase. Thus, a dose of atropine just sufficient to cause a 50% inhibition of salivation in the sensitive animals—giving an ED50 in quantal response—would produce a mean inhibition of considerably less than 50% on the graded response scale, when the almost negligible responses of the atropine-resistant animals are taken into account.

Cahen and Tvede eventually regarded the rabbit as an unsuitable test animal "because of the variability of the individual responses and its unusual tolerance to atropine." We have found, however, that rabbits without atropinesterase are very sensitive to atropine. In the rabbit the dose of atropine causing a 50% reduction in pilocarpine-induced salivation (3–7 μg./kg. s.c.) is considerably less than that needed in the mouse for a 50% maximal increase in pupil size (100 μg./kg. s.c.) and similar to that causing a 50% inhibition of the hypotensive response to a just-maximal dose of acetylcholine in the cat (1 μg./kg. i.v.) (Brown, Quinton, Acred, Bainbridge, and Turner—unpublished observation). Lands *et al.* (1946) also quote a low dose (1 μg./kg. i.v.) for a 70% inhibition of pilocarpine-induced salivation in rabbits.

Certain workers have administered atropine intravenously (Lands *et al.*, 1946; Tripod, 1949; Chen, 1954; Luduena and Lands, 1954). Atropinesterase may not have time seriously to affect the action of atropine given in this way, particularly when, as in the technique of Luduena and Lands, the salivary flow is measured for two periods of only 10 min. each, immediately before and after the injection of atropine. This point was therefore investigated in two sets of eight rabbits. In the first set, composed entirely of atropine-sensitive animals, the mean salivary response in the 10 min. after an intravenous injection of 10 μg./kg. atropine was 6.0±2.0% (S.E.) expressed as a percentage of the amount of saliva obtained in the preceding 10 min. In the other set of animals, all of which possessed atropinesterase in their blood, the corresponding figures were 63.6± 12.3% (S.E.). Pilocarpine nitrate (1 mg./kg.) was given to both groups 15 min. before the atropine. The difference between the mean salivary responses for the two groups is highly significant. It appears therefore that even with intravenous administration of atropine it is still necessary to use atropine-sensitive rabbits, since the atropinesterase affects the activity of a low dose of atropine even within 10 min. of intravenous injection.

In order to minimize variation between different rabbits, drugs were compared in the same animals. By the use of a (2+2) or (2+2+2) design whereby each of a set of eight rabbits received each of two dose-levels of the drugs tested, the relative potency in terms of a standard has been obtained with its limits of error ($P=0.95$). The values for λ (the standard deviation of a single log dose) ranged from 0.20 to 0.40. We found the relative potencies of atropine methyl nitrate and homatropine in terms of atropine to be 2.3 and 0.009 respectively. Issekutz (1917) reported that atropine methyl nitrate was 3 to 4 times, and homatropine about one fifth, as potent as atropine,

whereas according to Luduena and Lands (1954) the methyl nitrate was eight times as potent as atropine on intravenous administration. It is possible that slowness of absorption of the quaternary compound after subcutaneous injection may partly account for the lower potency obtained by us.

We have not found any reports on the activity of tricyclamol, oxyphenonium, and propantheline in antagonizing pilocarpine-induced salivation in rabbits, although Drill (1954) quotes from some unpublished data supplied by Clark and co-workers that "the intrinsic anti-salivary activity (of oxyphenonium) assayed in rabbits is 1.85 times greater than that of atropine." We have found the relative potency of tricyclamol to be 0.62. The approximate relative potencies for oxyphenonium and propantheline, derived by comparing ED50 values from responses in the same rabbits but not at the same times, were 0.9 and 1.1 respectively.

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GANGLION-BLOCKING PROPERTIES OF ATROPINE-LIKE DRUGS

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J. G. BAINBRIDGE AND D. M. BROWN

From the Beecham Research Laboratories, Ltd., Brockham Park, Betchworth, Surrey

(RECEIVED OCTOBER 28, 1959)

The ganglion-blocking properties of atropine, atropine methyl nitrate, oxyphenonium, poldine, methantheline and propantheline have been examined and compared with those of hexamethonium, mecamylamine, pentolinium and tetraethylammonium. The ganglion-blocking activity was assessed by stimulating the preganglionic nerve to the superior cervical ganglion and recording directly the percentage depression in the postganglionic action potentials. In this way any ambiguity due to the peripheral actions of the drugs was removed. As a group the atropine-like compounds are not markedly less potent than the ganglion-blocking agents, but their action is relatively transient. This work suggests that the ganglion-blocking action of atropine-like substances plays no part in their therapeutic effects.

Atropine-like drugs are frequently used in clinical practice for their peripheral anti-acetylcholine effects. The actions of atropine-like drugs at peripheral sites have been fully investigated, but their actions at autonomic ganglia have been examined in less detail [Marrazzi (1939), Dutta (1949), Konzett and Rothlin (1949), Ambache (1949), Cahen and Tvede (1952), and Paton (1954)]. We therefore thought it important to find out whether these drugs produce significant ganglion-block in doses which antagonize the peripheral actions of acetylcholine.

The measurement of ganglion-blocking action has usually been carried out on the cat nictitating membrane preparation, but there are several objections to this, particularly for atropine-like drugs which have a considerable peripheral action. For example, Cervoni, West, and Fink (1956) found that atropine directly depressed the smooth muscle of the nictitating membrane. This was confirmed by Thompson (1958) using the isolated nictitating membrane preparation. Even direct perfusion of the ganglion with the drug does not eliminate this error, since up to 50% may not reach the ganglion, being diverted possibly by arterio-venous anastomoses to peripheral sites (Paton, 1954).

In this investigation we have avoided complications due to peripheral actions of the drugs by measuring directly the postganglionic nerve potentials evoked by stimulating the preganglionic nerve to the superior cervical ganglion. We have studied the ganglion-blocking activity of several atropine-like drugs and some true ganglion-blocking agents.

METHODS

Stimulation and recording of postganglionic action potentials were similar to the method described by Eccles (1935).

Anaesthesia was induced in cats with ether, followed by intravenous chloralose/urethane solution (1% and 5% respectively) at approximately 5 ml./kg. The cervical sympathetic nerve and the postganglionic nerve from the superior cervical ganglion were dissected out. Care was taken to leave the blood supply to the ganglion intact. The cat was then transferred to a metal screening cage, stimulating and recording electrodes were placed in position and the exposed tissues were covered with liquid paraffin.

A rectangular pulse of approximately 0.1 msec. duration, 2 to 10 V. and 2 pulses/sec. frequency was applied to the preganglionic nerve. The recording electrodes on the postganglionic nerve led to a condenser-coupled amplifier and oscilloscope with recording camera.

Drugs were given intravenously into the femoral vein. The action potentials were photographed immediately before and at 1 min. after the injection, the effect of the compounds being maximal at this time. The action of the drug was expressed as the percentage reduction of the spike amplitude as measured from the photographic record.

Four to eight observations were made with each drug. The response was plotted against the dose on a log scale and a regression line was fitted by eye to the points obtained. The ED50 was read directly from the graph.

The duration of action of the atropine-like drugs was determined by injecting a dose equal to the ED50 and photographing the action potential before administration and at 1, 2, 4, and 8 min. after administration. The action potentials following hexamethonium were measured at 5 min. intervals until recovery. The percentage depression of spike amplitude was plotted against time, and from the graph the time taken from maximum depression to half recovery was estimated. Two experiments were carried out with each drug.

The following salts of the various drugs were used: pentolinium tartrate, hexamethonium bromide, mecamylamine hydrochloride, atropine sulphate, tetraethylammonium bromide, methantheline bromide, propantheline bromide, oxyphenonium bromide, and poldine methosulphate (Nacton).

The doses given below are expressed in terms of these salts.

Results

Fig. 1 illustrates the action potentials before and after the administration of 0.2 mg./kg. of hexamethonium and 4 mg./kg. of atropine. This dose of atropine reduced the spike potential by approximately 80%, and hexamethonium by about 35%.

Fig. 2 shows the dose-response lines obtained for most of the drugs investigated. The slopes of the lines would appear to be all of the same order.

The doses of atropine-like drugs and ganglion-blocking agents giving a 50% reduction in spike amplitude, as determined from the dose-response lines, are given in Table I.

It will be noted that the atropine-like drugs, as a group, are somewhat less potent than the true ganglion-blocking agents, but the difference is not marked. In the case of atropine methyl nitrate this drug is even more potent than mecamylamine and hexamethonium. However, the two groups of drugs differ considerably in their duration of action. The atropine-like drugs have a short effect of approximately 2 to 6 min., while the duration of action of the true ganglion-blocking drugs administered at the ED50 level is upwards of 30 min.

A comparison of the duration of action of atropine-like drugs with hexamethonium was made and the results are given in Table II. As the time of complete recovery was rather variable, and as the initial slope of the recovery curve was more constant, the " mean half recovery time " as defined above was worked out.

Table I

RELATIVE GANGLION-BLOCKING POTENCY OF ATROPINE-LIKE DRUGS AND TRUE GANGLION-BLOCKING AGENTS WITH RESPECT TO HEXAMETHONIUM

Drug	ED50 (mg./kg.)	Equipotent Molar Ratio (Hexamethonium = 1.0)
Pentolinium	0·07	6·55
Mecamylamine ..	0·23	0·733
Hexamethonium ..	0·31	1·0
Atropine methyl nitrate	0·14	2·22
Propantheline	0·70	0·545
Methantheline	0·70	0·510
Tetraethylammonium ..	0·80	0·226
Atropine	2·4	0·249
Oxyphenonium ..	2·4	0·154
Poldine (Nacton) ..	3·8	0·097

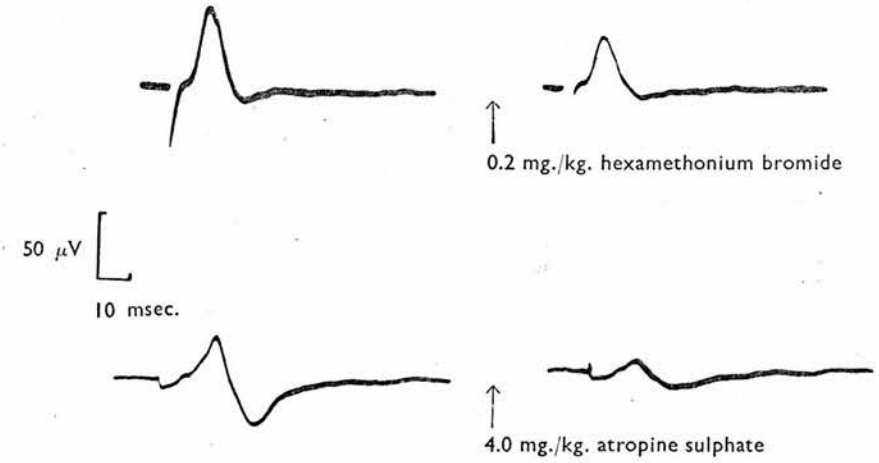

FIG. 1.—Postganglionic action potentials. Top records: before and after the intravenous injection of 0.2 mg./kg. of hexamethonium. Bottom records: before and after 4 mg./kg. of atropine.

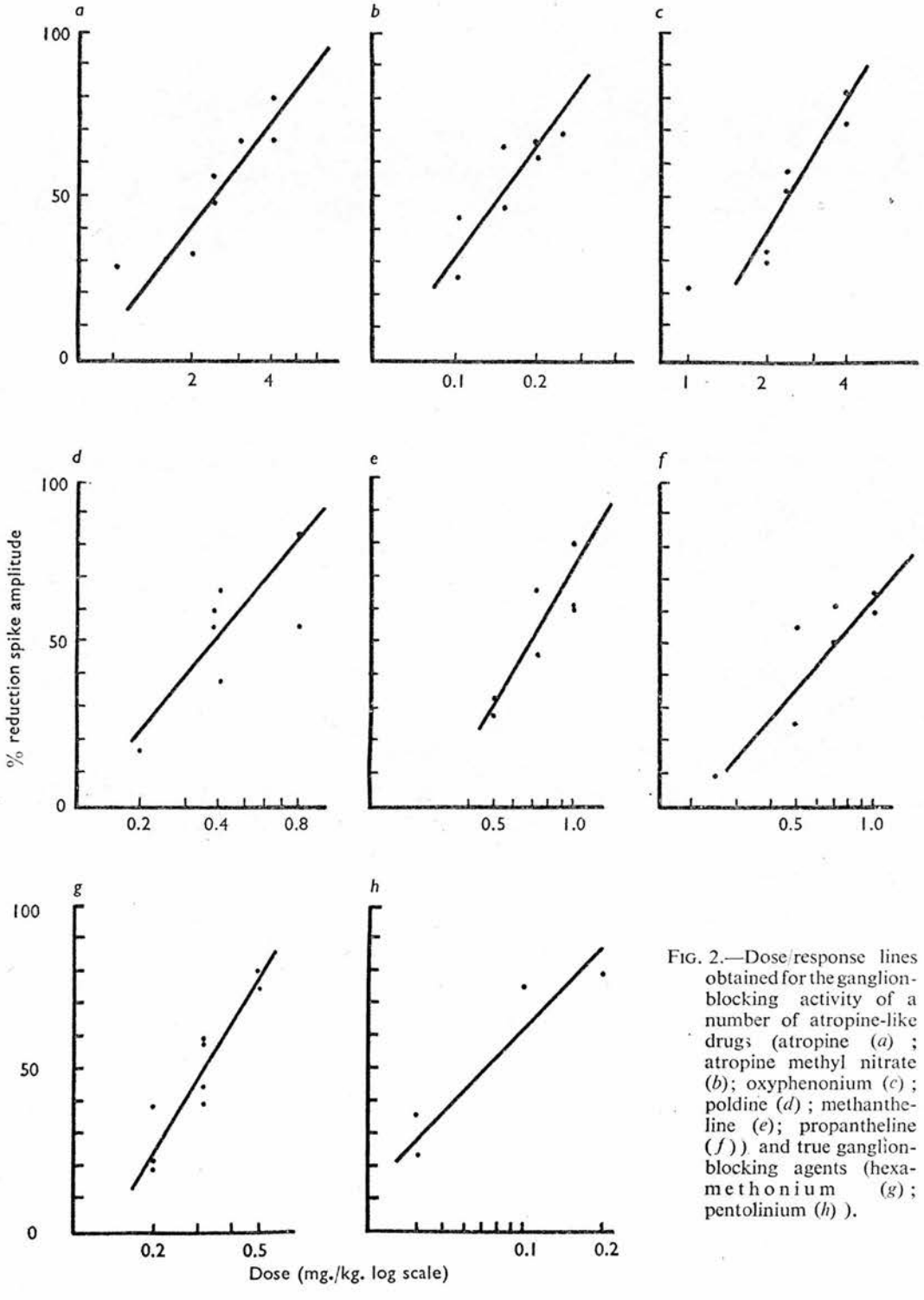

FIG. 2.—Dose/response lines obtained for the ganglion-blocking activity of a number of atropine-like drugs (atropine (*a*) ; atropine methyl nitrate (*b*); oxyphenonium (*c*) ; poldine (*d*) ; methantheline (*e*); propantheline (*f*)) and true ganglion-blocking agents (hexamethonium (*g*) ; pentolinium (*h*)).

TABLE II

TIME TAKEN FOR THE POSTGANGLIONIC ACTION POTENTIAL TO RECOVER BY 50% AFTER THE ADMINISTRATION OF THE ED50 OF SOME ATROPINE-LIKE DRUGS AND HEXAMETHONIUM

Drug	Mean Half Recovery Time (Min.)
Oxyphenonium	5·5
Atropine	4·2
Atropine methyl nitrate	4·7
Poldine	5·8
Propantheline	3·3
Methantheline	2·3
Hexamethonium	31·0

DISCUSSION

Atropine and various anti-acetylcholine drugs have been shown by a direct method to block transmission through the superior cervical ganglion.

Fig. 3*a* shows the relation between toxic, ganglion-blocking, and peripheral anti-acetylcholine activity of the atropine-like drugs, and Fig. 3*b* shows the relationship between the toxic and ganglion-blocking doses for the true ganglion-blocking drugs. The toxic dose is the intravenous LD50 for mice, and the figure for anti-acetylcholine activity is the dose required to obtain 50% inhibition of the vasodepressor response to intravenous acetylcholine in the cat (Acred, Atkins, Bainbridge, Brown, Quinton, and Turner, 1957). The ganglion-blocking dose is the ED50 value as given in Table I.

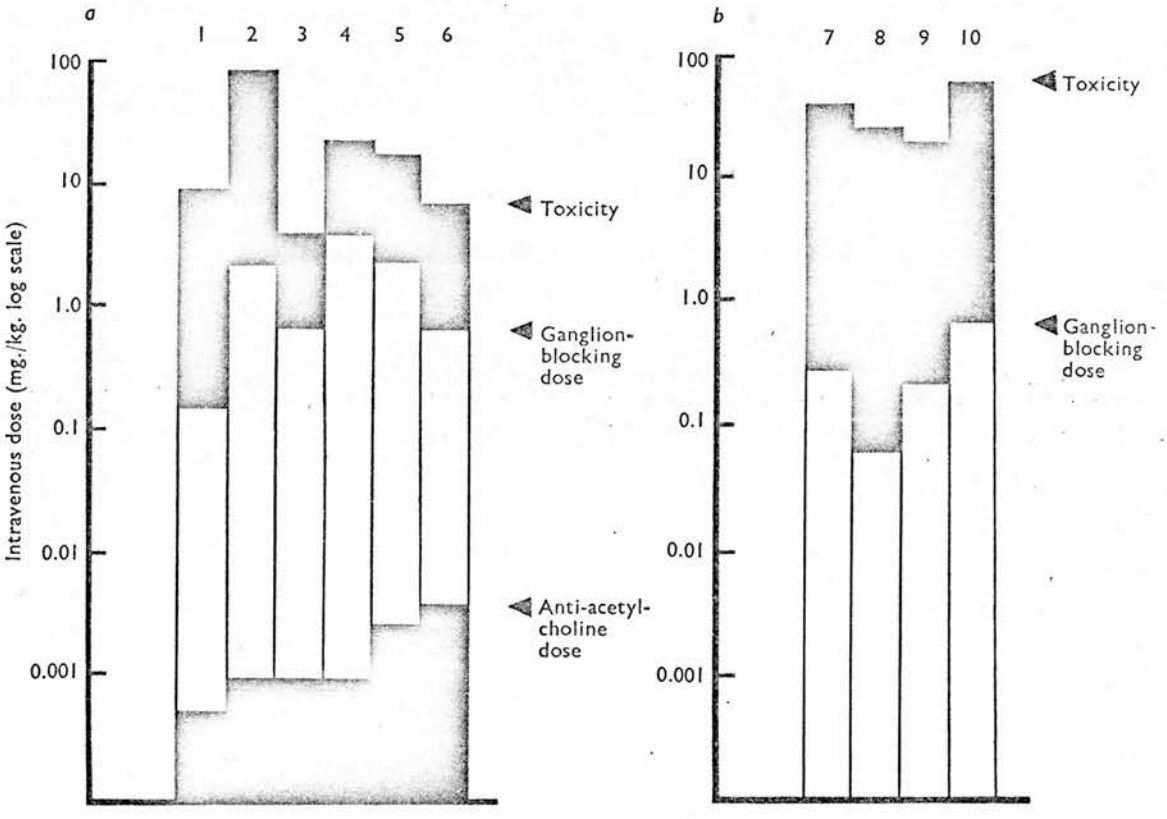

FIG. 3.—Diagram showing (*a*) relationship between toxicity, ganglionic and peripheral activities of atropine-like compounds (1, atropine methyl nitrate; 2, atropine; 3, propantheline; 4, poldine; 5, oxyphenonium; 6, methantheline); and (*b*) toxic and ganglionic activity of ganglion-blocking agents (7, hexamethonium; 8, pentolinium; 9, mecamylamine; 10, tetraethylammonium bromide).

In the case of the ganglion-blocking agents the ratio between toxic and ganglion-blocking doses is large, ranging from 400 for pentolinium to 90 for mecamylamine and tetraethylammonium, whereas in the atropine-like group the ratio varies from 6 for propantheline to 60 for atropine methyl nitrate. ED50 values for the ganglion-blocking activity for the two groups actually overlap, atropine methyl nitrate being remarkably potent whereas tetraethylammonium is less potent than methantheline and propantheline. The ratio between ganglionic and peripheral ED50 values in the atropine-like group is of the order of 1,000, and so it is most improbable that ganglion-block contributes to their therapeutic actions.

No simple relation exists between ganglionic and peripheral potencies in the atropine group. Quaternization of the nitrogen in atropine leads to an increase of toxicity and peripheral anti-acetylcholine activity and to a proportionately much greater ganglion-blocking potency. The presence of a quaternary nitrogen atom is, however, not essential to a high ganglion-blocking potency (for example, mecamylamine and pempidine).

The effect of anti-acetylcholine drugs at ganglia could be due to depolarization or competition with acetylcholine. Paton and Perry (1953) have shown that acetylcholine, nicotine, and tetramethylammonium block ganglia by depolarization, all causing an initial stimulation of the ganglion cells. Other known ganglion-blocking agents neither depolarize nor cause an initial stimulation and are assumed to act competitively. We have no evidence that the atropine-like drugs cause an initial stimulation of the superior cervical ganglion, although we did produce an initial stimulation of the intestine *in vivo* after intravenous administration (Acred *et al.*, 1957). It is possible that this is a nicotine-like effect. On the other hand, Konzett and Rothlin (1949), perfusing the superior cervical ganglion and recording from the nictitating membrane, overcame the block produced by atropine with higher concentrations of acetylcholine, suggesting a competitive mode of action.

The ganglion-blocking action of atropine can be attributed partly to its local anaesthetic activity (Paton, 1954). However, most of the compounds we have investigated are quaternary esters and have no local anaesthetic properties (Acred *et al.*, 1957; Barlow, 1955). Further, while atropine has about half the local anaesthetic potency of procaine the latter has a much smaller ganglion-blocking action than atropine both by intravenous and close intra-arterial injection to the ganglion (see also Dutta, 1949). Hence the available evidence as to mode of action suggests a competitive block such as probably occurs at peripheral sites, although other modes of action are possible (Paton, 1954).

Although the ganglionic action of atropine could hardly become manifest in clinical use it could explain the observation that in anaesthetized cats intravenous doses greater than 1 mg./kg. often cause a transient fall of blood pressure.

We can conclude that the ganglion-blocking action of atropine-like drugs does not add significantly to their peripheral actions. In the first instance, the dose required to produce a ganglion-block is so great that the degree of side-effects alone would prohibit their use. In the second instance, the duration of block is so short that in no way could it be said to influence the therapeutic action. Finally, the precise mechanism of the block has not as yet been elucidated.

The authors wish to thank Miss A. Trenchard for technical assistance.

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THE PHARMACOLOGY OF (1-METHYL-2-PYRROLID-2-YL) METHYL PHENYLCYCLOHEXYL GLYCOLLATE METHOBROMIDE (BRL. 556)

BY

D. M. BROWN, D. E. HALL AND D. H. TURNER

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ACRED, ATKINS, BAINBRIDGE, BROWN, QUINTON and TURNER (1) have shown that esters of 1-alkyl-2-hydroxyalkylpyrrolidine and their quaternary derivatives possess considerable atropine-like activity. None of the compounds examined, however, had an activity which significantly exceeded that of atropine sulphate either in potency or duration. The pharmacological properties of a new derivative, (1-methyl-2-pyrrolid-2-yl) methyl phenylcyclohexyl glycollate methobromide (BRL. 556) are described in this paper. The compound has a strikingly long duration of action *in vivo*.

METHODS

Tests in Vitro

Anti-acetylcholine action. (*a*) Segments, approximately 2 cm long, were removed from the lower ileum of young guinea pigs weighing not more than 250 g and suspended in oxygenated Tyrode solution at 37° in a 5 ml bath. Acetylcholine (ACh), which was allowed to act for 30 sec, was added at 2 min intervals until constant responses were obtained. Doses of atropine sulphate and test compound were added 30 sec before the ACh and were adjusted so that the ACh response was reduced to approximately 30 % and 70 % of the original. Between the successive doses of antagonist the ACh response was allowed to return to normal. A 16-point assay was then performed using a Latin square design (10) and the results analysed by the procedure of SCHILD (15).

(*b*) A further estimate of anti-ACh activity was obtained on the isolated guinea pig ileum preparation by determination of the pA_2 value as described by SCHILD (16). When the responses to a submaximal dose of ACh became constant the normal Tyrode solution was replaced by Tyrode containing a known concentration of antagonist. At the same time the dose of ACh was doubled. A 2 min cycle with a contact time of 30 sec was used for the ACh. Dosing continued for 14 min. Three concentrations of antagonists were used and for each test a fresh piece of gut was employed.

Antihistamine action. Preparations of guinea pig ileum were made as for the anti-ACh test. Histamine was added at 90 sec intervals until the responses were regular. The antagonist was then added to the bath 30 sec before the histamine and the depression in response compared with that obtained by a standard antihistamine drug (antazoline sulphate).

Curare-like activity. The rate phrenic nerve-diaphragm preparation, as described by BULBRING (7), was used.

Tests in Vivo

Acute toxicity. Acute oral, subcutaneous and intravenous toxicities were determined in mice of either sex, weighing 17–23 g. For the intravenous toxicity doses were given into the tail vein in 5 sec. The dose volume used throughout was 1 ml/100 g. Ten mice were used at each of three dose levels and the number of deaths within 30 min recorded for the intravenous toxicity, and at 24 hr for the subcutaneous and oral toxicities. The results were analysed by the method of FINNEY (9).

Anti-acetylcholine activity on cat blood pressure. Cats were anaesthetised with ether followed by intravenous chloralose/urethane (0.7 %. w/v chloralose, 2.8 % w/v urethane, approximately 8 ml/kg). A tracheal cannula was inserted and blood pressure recordings made from the carotid artery which was cannulated and connected to a mercury manometer. Injections were made via a polythene cannula inserted in the saphenous vein. A submaximal dose of ACh (1–5 μg) was given intravenously at 2 min intervals until a regular depression of blood pressure had been obtained. Atropine sulphate and BRL. 556 were given intravenously at 1 min precontact to ACh and low and high doses selected, such that approximately 20 % and 80 % inhibition of the depressor response was produced. After each dose of antagonist, further doses of ACh were given until a normal or constant response was obtained. A2 × 2 Latin square design assay (10) of BRL. 556 against atropine

sulphate was carried out. The results were subjected to standard statistical procedure (15).

Ganglionic block. The action of BRL. 556 on autonomic ganglia was examined in the superior cervical ganglia of the cat by the method described by BAINBRIDGE and BROWN (3).

Mydriatic activity and duration of action.

(*a*) *Mice.* An estimation of mydriatic activity was carried out in the mouse according to the method of PULEWKA, as used by ING, DAWES and WAJDA (12). Three doses of each compound were given subcutaneously using a dose ratio of 1 : 2. Ten mice, 16 to 24 g, were allocated to each dose and the pupil diameter was measured in arbitrary units with the aid of a micrometer scale set in the eyepiece of a dissecting microscope. The pupil diameter was measured at 10 min intervals over a period of 140 min after dosing. Atropine sulphate was used as a standard. The relative activity of BRL. 556 was estimated at 20 min after dosing and the results were analysed according to the procedure described by BLISS and MARKS (4).

(*b*) *Rats.* Rats of either sex, weighing 100 to 200 g, were used. After measurement of the initial pupil diameter BRL. 556 and atropine sulphate were given at a dose level of 20 mg/kg subcutaneously to each of 3 rats. The pupil diameter was measured at various intervals after administration.

Salivary flow.

(*a*) The effect of BRL. 556 on salivary flow was estimated by the method described by BROWN and QUINTON (5).

(*b*) A further estimate of the effect of BRL. 556 was obtained in the cat by the technique described by BÜLBRING and DAWES (8). Cats were anaesthetised with ether followed by intravenous chloralose/urethane mixture (8 ml/kg : 0.7 % chloralose, 2.8 % urethane). Whartons duct was cannulated with polythene tubing (Allen and Hanbury No. 1). The cannula was connected to a water displacement bottle, which in turn was connected to a drop tube. The number of drops was recorded by a Thorpe impulse counter. Continuous intravenous infusion of carbachol/adrenaline solution was made into the saphenous vein by a Palmer constant rate infusion apparatus. Infusion solutions were made up in normal saline containing 4 % w/v ascorbic acid. Satisfactory rates of salivary flow were obtained with initial doses of 4 to 8 μg/min carbachol and 2 to 4 μg/min adrenaline, but as the preparation became less sensitive the final doses after about 2 hr were 10 to 20 μg/min

carbachol and 5 to 10 μg/min adrenaline. A 2 × 2 Latin square design assay (10) of atropine sulphate against BRL. 556 was carried out. The drugs were given intravenously. The results were subjected to standard statistical procedure (15).

Gastric secretion. The activity of BRL. 556 in relation to atropine sulphate was assessed by measuring the inhibition in volume of gastric secretion in the 2 and 6 hr. Shay rat preparation by the method described by BROWN and TURNER (6). In addition the mydriatic effect of the compounds was estimated during the course of the assay, the response being scored as follows :

75–100 % dilatation — 4
50–75 % dilatation — 3
25–50 % dilatation — 2
5–25 % dilatation — 1

Pancreatic secretion. A test for inhibitory action on pancreatic secretion was carried out on the lines of the starch tolerance test used by ALTHAUSEN and UYEYAMA (2). Rabbits, 2 to 3 kg (male or non-pregnant females) were used. The rabbits were fasted for 24 hr during which period water was allowed *ad lib*, and were rested quietly for at least half an hour before commencement of an experiment. An initial blood sample was taken from the marginal ear vein (fasting level) and then at intervals of one hour up to 5 hr after the administration, by stomach tube, of starch, glucose or water (control). The starch meal, per animal, consisted of 5 g soluble starch in 20 ml distilled water containing 1.0 ml N/10 HCl. The starch was partially hydrolysed by boiling for 10 min after which the meal was allowed to cool to 40° C, the temperature at which it was administered. 20 ml 50 % w/v glucose in distilled water was used as the glucose meal. The administration of the starch and glucose was followed by 10 ml of distilled water. The control consisted of 30 ml distilled water. BRL. 556 was administered intravenously 1 hr before giving the starch, glucose or water. Blood sugar determinations were carried out by the method of HAGEDORN and JENSEN (11) and plasma amylase estimated by the method of KING and WOOTTON (13).

Absorption from the gastro-intestinal tract. The absorption of BRL. 556 and atropine sulphate was assessed in the Shay rat by estimating the pupil dilatation produced at 1 ½, 2 ½, 4 and 6 hr after dosing. Four rats were used at each dose level and 4 control rats which received normal saline were set up at the same time. Pupil dilatation was scored by the method used in the gastric secretion experiments. The drugs

were dissolved in normal saline and the dose volume used throughout was 1 ml/100 g.

(*a*) *Intragastric dosing*. Doses were administered immediately the rats regained consciousness after pyloric ligation.

(*b*) *Intra-intestinal dosing*. After ligating the pylorus a loop of nylon thread was placed around the duodenum about 2 cm below the pylorus. With the aid of a fine cautery a small aperture was made into the duodenum between the pylorus and the loop. A cannula consisting of about 3 cm polythene tubing attached to a hypodermic needle was passed down the duodenum and the thread tied round it. The dose was then administered, the cannula slowly withdrawn and the thread tied simultaneously.

Results

Anti-acetylcholine, antihistamine and curare-like activity in vitro. BRL. 556 was found to possess no significant antihistamine or curare-like activity. When anti-ACh activity was assessed at a precontact period of 30 sec BRL. 556 was found to have anti-activity of 0.77 times that of atropine sulphate. pA_2 determinations for anti-ACh activity at a 14 min contact period gave values of 9.0 and 8.6 for BRL. 556 and atropine sulphate respectively, representing an activity for BRL. 556 of 2.5 times atropine sulphate.

Acute Toxicity. The following LD_{50} (with 5 % fiducial limits) values for BRL. 556 were obtained in mice: intravenous 18.0 (16.8 to 19.2) mg/kg, subcutaneous 303.0 (289.1 to 317.0) mg/kg, and oral 1039.9 (626.7 to 1722.0) mg/kg.

Anti-acetylcholine activity on cat blood pressure. It was found that there was a decrease in sensitivity to acetylcholine and increase in sensitivity to the antagonists as the experiment proceeded, but constant responses to both were obtained after about 3 hr of dosing. Two complete assays were carried out and a mean relative potency of BRL. 556 of 0.4 times atropine sulphate was obtained. Recovery from doses of BRL. 556 was slower than from atropine sulphate.

Ganglionic block. At a dose level of 1.5 mg/kg intravenously BRL. 556 produced a 50 % reduction in post-ganglionic potential following stimulation of the pre-ganglionic nerve to the superior cervical ganglion. BRL. 556 was found to have 0.2 times the activity of hexamethonium bromide but it's duration of action was only 9.5 min compared to 31 min for hexamethonium at an equi-effective dose.

Mydriatic activity and duration of action.

(*a*) *Mice.* The mean pupil diameter of mice receiving atropine sulphate and BRL. 556 at various time intervals after subcutaneous administration with 0.05, 0.10 and 0.20 mg/kg are shown in Fig. 1.

At all dose levels maximum dilatation occurred after 20 min. After 140 min the pupil diameter of mice which had received atropine sulphate had returned to normal, but with mice which had received BRL. 556 there was still pronounced dilatation even with the lower dose of 0.05 mg/kg. BRL. 556 was 1.5 times as active as atropine sulphate at the time of maximal dilatation.

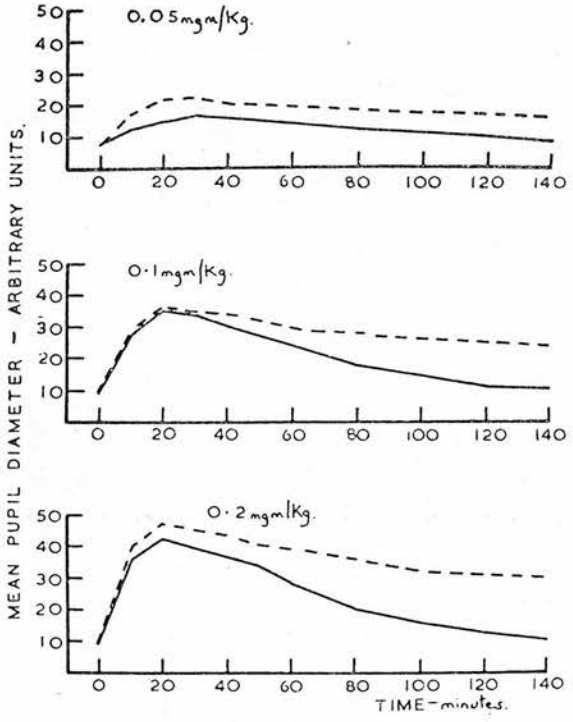

FIG. 1

Mydriatic activity of subcutaneously administered BRL. 556 (broken line) and atropine sulphate (continuous line) in groups of 10 mice.

(*b*) *Rats.* The pupil diameter at various time intervals after the subcutaneous administration of 20 mg/kg BRL. 556 and atropine sulphate were recorded until they had returned to the approximate pretreatment levels. Following atropine sulphate the pupil diameters had returned to normal within 27 hr, while following BRL. 556 they did not return to normal till 190 hr (8 days) after dosing (FIG. 2).

Salivary flow. The relative potency of BRL. 556 to atropine sulphate was determined in the rabbit at 15 min after administration of the drugs, and it was found that BRL. 556 was 3.0 times (5 % limits of error of 1.8 to 4.8 mg/kg) as active as atropine sulphate.

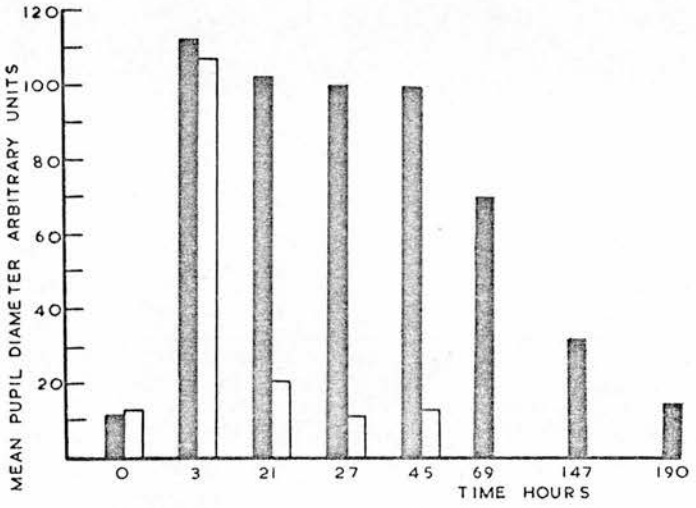

FIG. 2

Mydriatic activity in groups of 3 rats receiving 20 mg/kg subcutaneously of either BRL. 556 (black columns) or atropine sulphate (blank columns).

In the cat, however, where the activity was assessed immediately following the administration of the drug, it was found that the relative potency of BRL. 556 in terms of atropine sulphate was 1.5 (5 % limits of error of 1.2 to 2.0 mg/kg).

Gastric secretion. The results of the effect of BRL. 556 and atropine sulphate are shown in Fig. 3.

For the 2 hr Shay rat BRL. 556 was found to be 7.2 times as active as atropine sulphate (5 % limits of error of 5.0 to 10.6 mg/kg). The pupils of the rats in this experiment were considerably dilated with atropine sulphate at the high dose, although there was only slight dilatation at the low dose. On the other hand, the rats receiving BRL. 556 showed negligible dilatation of the pupil at the high dose and no detectable dilatation at the low dose. In the 6 hr Shay rat the relative potency of BRL. 556 to atropine sulphate was 37.6 (limits of error of 12.4 to 114.0 mg/kg). During the course of the experiments at the 6 hr period the high dose of BRL. 556 showed comparable dilatation of the pupil to atropine sulphate, but at the low dose the dilatation was much less with BRL. 556 although by the end of the forth hour of the test the

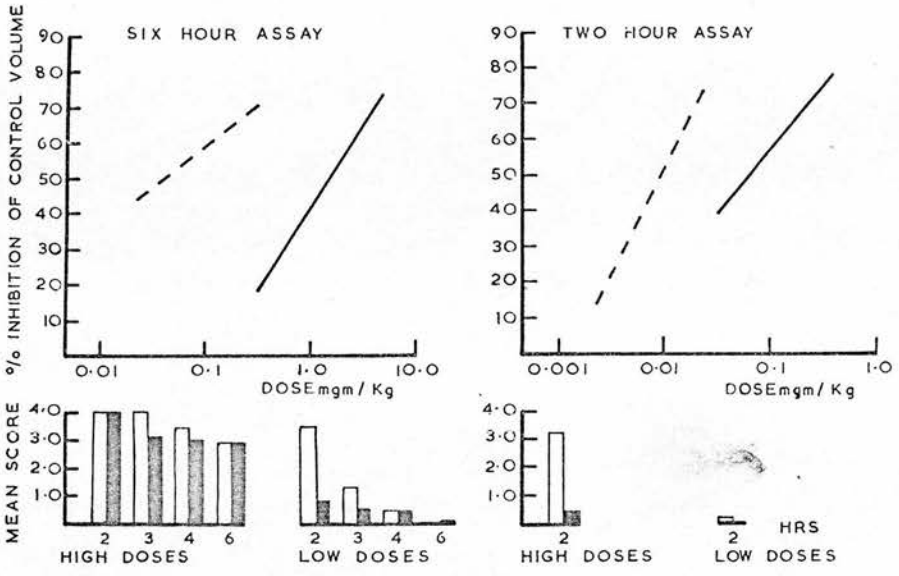

FIG. 3

Dose response lines for BRL. 556 (broken line) and atropine sulphate (continuous line) in the 2 and 6 hr pyloric ligated rat. Columns represent the mean pupil diameter for BRL. 556 (black) and atropine sulphate (blank) during the course of the assays.

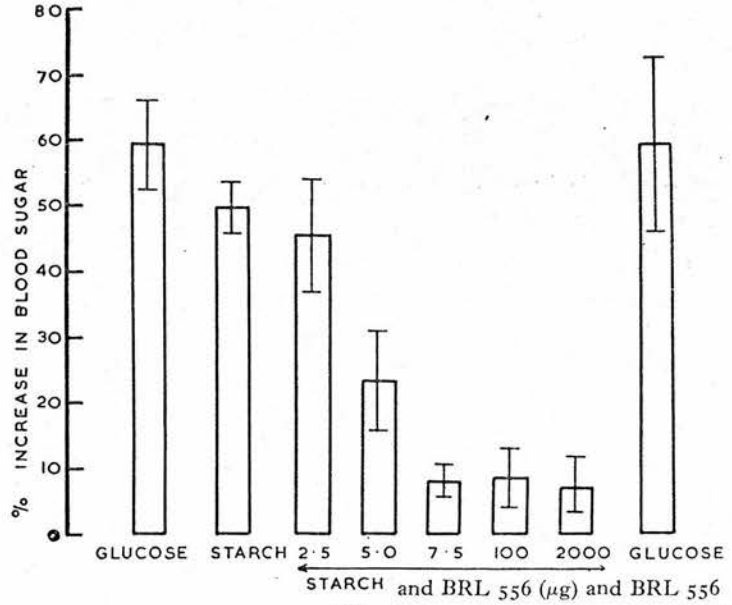

FIG. 4

Effect of pretreatment with BRL 556 on the elevation of blood sugar produced by orally administered starch. Each column represents the mean of four observations with the standard error of the mean.

effect of atropine sulphate had worn off and the pupil dilatation was not greater than that seen with BRL. 556.

Pancreatic secretion. The effect of BRL. 556 on the blood sugar picture of rabbits following a starch meal is illustrated in Fig. 4.

BRL. 556 within the dose range of 2.5 to 7.5 μg/kg intravenously caused a reduction in blood sugar which was proportional to the dose, but with dose of 7.5 μg/kg and greater a complete block of the response to the starch meal was not obtained. BRL. 556, 2 mg/kg intravenously, had no effect on the blood sugar level following administration of glucose. The serum amylase of plasma of control rabbits, and rabbits receiving 2 mg/kg BRL. 556 intravenously did not differ significantly. Levels ranged between 400 to 500 Somagyi units/100 ml plasma in both groups.

Absorption from the gastro-intestinal tract. Pupil dilatation after intra-intestinal and intra-gastric administration of 1.0, 5.0 and 10.0 mg/kg BRL. 556 and atropine sulphate is shown in Fig. 5. Intestinal absorption of atropine sulphate occurred at a dose level of 1.0 mg/kg. However, significant absorption from the stomach only occurred with a dose of

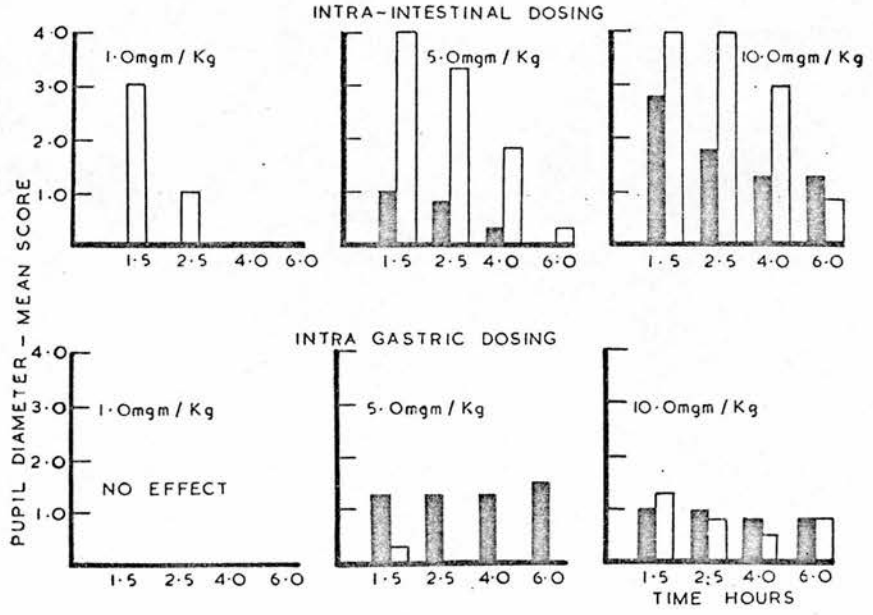

FIG. 5

Absorption of BRL. 556 (black columns) and atropine sulphate (blank columns) from the intestine and stomach of the pyloric ligated rat. Each column represents the mean of four observations.

10 mg/kg. With BRL. 556 there was a greater absorption from the stomach than the intestine following a dose of 5 mg/kg but when the dose was increased to 10 mg/kg there was more absorbed from the intestine.

Discussion

BRL. 556 is a potent anti-ACh compound but it's relative potency to atropine sulphate both *in vitro* and *in vivo* is dependent on the method of assessment. On the isolated guinea pig ileum with a pre-contact time of 30 sec, the anti-ACh activity of BRL. 556 was 0.77 times that of atropine sulphate, whereas in the determination of the pA_2 value, where the pre-contact time was 14 min, BRL. 556 had a relative potency of 2.5.

In *in vivo* experiments where the activity of the drug is assessed within periods up to 20 min of administration, i.e. the inhibition of the vasodepressor response to ACh, the inhibition of salivation as measured by cannulation of the Wharton's duct and the mydriatic response in mice, the activity of BRL. 556 was of the same order as atropine sulphate. However, in the Shay rat test when the assessment of activity was carried out at 2 and 6 hr after administration of the compounds, the relative activity of BRL. 556 to atropine sulphate rose from 7.2 to 37.5 respectively.

In addition to its effect on gastric secretion, BRL. 556 had a pronounced action on salivation and pancreatic secretion. With regard to the latter, there was complete block of the vagal component in doses above 7.5 μg/kg intravenously leaving the basal secretion unaffected.

Biochemical studies (Mansford, personal communication) have shown that 28 % of the injected dose of BRL. 556 is excreted within 24 hr. of administration, and that the close analogue poldine methosulphate ([1-methyl-2-pyrrolid-2-yl] methyl benzilate methosulphate) is excreted at the same rate. Poldine methosulphate has a similar duration of action to atropine sulphate (6) and therefore the enhanced duration of action *in vivo* may be accounted for by a firmer binding to the cholinergic receptor site and is not due to a difference in rate of elimination.

According to observations made by Schanker (14) only weak bases which are dissociated in acid solutions are absorbed by the stomach and in the intestine, weaker bases are preferentially absorbed to stronger bases. As regards intestinal absorption we have found that our results agree with these observations. However, in the stomach we have found that the stronger base, BRL. 556 is absorbed more readily than the weaker base, atropine sulphate.

From previous studies we have noted that the phenyl-cyclohexyl glycollic acid moiety is responsible for the duration of action. For example, oxyphenonium [diethyl(2-hydroxyethyl) methyl ammonium bromide α-phenylhexyl glycollate] is 7.6 times as active as atropine sulphate in the 6 hr Shay rat. As far as is known no other acid moiety has been shown to be responsible for such prolonged activity.

Summary

BRL. 556 (1-methyl-2-pyrrolid-2-yl) methyl phenylcyclohexyl glycollate, has been found to be a potent atropine-like compound with a long duration of action.

In the pyloric ligated (Shay) rat, relative potencies, in terms of atropine sulphate (= 1.0), of 7.2 and 37.5 were obtained in the 2 and 6 hr preparation respectively. Other *in vivo* tests such as the inhibition of vasodepressor response to acetylcholine, the inhibition of salivation, and the mydriatic response, have shown the activity of BRL. 556 to be of the same order of activity as atropine sulphate. *In vitro* anti-acetylcholine activity as assessed at 30 sec contact and 14 min contact (pA_2 determination) have given relative potencies of 0.7 and 2.3 respectively. The differences in the relative potency obtained by the various methods is discussed.

BRL. 556 has no curare-like or antihistamine properties.

Acknowledgments

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BRL 1288—A NEW ANTI-PARKINSON DRUG

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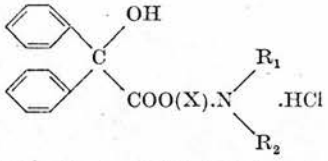
BRL 1288—a New Anti-Parkinson Drug

THE symptomatic treatment of Parkinson's disease usually involves anti-acetylcholine drugs which may block muscarinic receptor sites on neurones in the reticular formation. Unfortunately, all of the drugs in common use have pronounced peripheral anti-acetylcholine actions which result in unpleasant side effects, such as dryness of the mouth and mydriasis.

Doyle *et al.*[1] have described the pharmacology and chemistry of a series of basic esters and have prepared glycollic acid esters derived from the open chain alcohols by transesterification of methyl α,α-diphenyl-glycollate with an appropriate amino alcohol to form basic esters of the general formula



(X = alkylene group containing two or three carbon atoms)

R_1 and R_2 = alkyl groups containing one to six carbons

While examining this series of compounds for anti-Parkinson activity, we found that the compound 2(ethyl-*n*-propylamino)ethyl α,α-diphenyl-glycollate hydrochloride[2] (BRL 1288) had maximum activity in the CNS and minimal peripheral anti-acetylcholine activity. In mice a dose less than one fifth of that which caused mydriasis reduced tremor. Any other anti-Parkinson drug would not inhibit tremor until doses, at least six times greater than those required to produce mydriasis, were given.

A modification of the method described by Everett, Blockus and Shepperd[3] was used to assess anti-Parkinson activity. These authors found that tremorine (1 : 4 dipyrrolidine but-2-yne) produces a generalized tremor and akinesia in mice, and that these effects can be antagonized by drugs having anti-Parkinson activity. Subsequently, it was shown[4] that the metabolite of tremorine, oxo-

Table 1. ANTI-OXOTREMORINE ACTIVITY IN MICE

Compound	Route	Time of assessment (min)	ED_{50} mg/kg
BRL 1288	p.o.	40	39
	s.c.	20	3·8
Benzhexol	p.o.	40	30
	s.c.	20	3·8

tremorine, was the active tremorogenic agent when given orally or subcutaneously.

We induced tremors in mice by the intraperitoneal injection of oxotremorine (2 mg/kg), and an observer, who was unaware of the treatment programme, assessed them subjectively on a scale of 0, 1 or 2 according to the degree of tremor.

Maximum tremor occurs approximately 10 min after oxotremorine injection and therefore the injection of BRL 1288 and of the reference drug, benzhexol, was timed so that the peak anti-tremor activity would coincide with the time of maximum tremor. BRL 1288 and benzhexol were given orally 40 min or subcutaneously 20 min before this peak. Ten female mice (SAS/ICI strain) weighing 18–22 g were allotted to each group. A control group, which received only oxotremorine, was included. Reductions in tremor in the test groups were expressed as a percentage of the control group and the results plotted on log probit paper. The ED_{50} values (mg/kg) were read from this graph (see Table 1).

To assess peripheral anti-acetylcholine activity *in vivo* the compounds were injected subcutaneously to mice kept under subdued lighting. Pupil diameters were measured on an arbitrary scale using a binocular dissecting microscope with a focal light source directed on the eye. Measurements were taken before and at suitable intervals after drug administration. The potency of BRL 1288 relative to benzhexol was 0·036, and Fig. 1 shows the time course of the mydriatic activity.

Further pharmacological studies have shown that the compound possesses anti-convulsant activity against maximal electroshock seizures in mice. An ED_{50} of 10·6 mg/kg was obtained 0·5 h after subcutaneous administration of BRL 1288 compared with an ED_{50} of 17·0 mg/kg for sodium phenobarbitone. Sedation occurs with large doses, but no other significant effects have been detected.

Rats absorb almost all the ^{14}C BRL 1288 given orally and no unchanged BRL 1288 can be detected in urine or faeces. Approximately 25 per cent of the radioactivity was excreted in the urine; the remainder, as a result of biliary excretion, went in the faeces. After giving unlabelled BRL 1288, benzilic acid, propylaminoethanol, ethylaminoethanol and ethylpropylaminoethanol have been identified by chromatography in urine. Humans excrete similar metabolites in the urine. Whole body

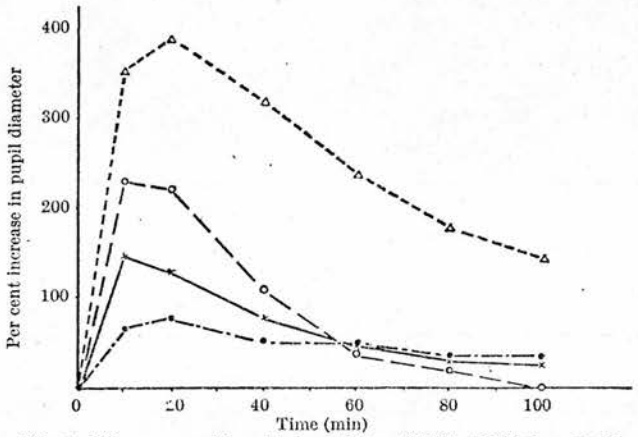


Fig. 1. Time course of mydriatic activity of BRL 1288 (○ - - ○, 20 mg/kg; ×—×, 10 mg/kg) and benzhexol (△ - - △, 0·8 mg/kg; ●— · —●, 0·4 mg/kg) in mice subcutaneously.

autoradiography of mice has shown that radioactivity is present in the brain, and preliminary chromatographic examination of mouse brain homogenates has revealed the presence of unchanged BRL 1288.

Initial toxicity studies have been carried out in the rat and dog. Toxic symptoms appear in rats injected subcutaneously with daily doses in excess of 150 mg/kg and there is evidence of hepatotoxicity. In the dog slight convulsions were observed after daily oral doses of 50 mg/kg, and lower doses were sedative.

Preliminary clinical studies with Parkinson patients at St Thomas's Hospital made in collaboration with Dr Marsden of the MRC Medical Unit, Department of Medicine and Neurology, St Thomas's Hospital, have shown that BRL 1288 is of considerable value in the symptomatic treatment of Parkinson's disease; peripheral anti-acetylcholine side effects were absent at effective clinical doses and the compound has not caused psychotoxic effects.

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THE METABOLISM AND DISTRIBUTION OF BENAPRYZINE HYDROCHLORIDE

(BRL 1288)

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METABOLISM AND DISTRIBUTION OF BENAPRYZINE HYDROCHLORIDE

(BRL 1288)

Abstract - Metabolism studies in rats with ^{14}C-labelled Benapryzine hydrochloride showed that the drug was well absorbed from the gut after an oral dose and that the bile was the most important route of excretion. No unchanged drug was detected in the urine, faeces or bile and the major route of metabolism was found to be hydrolysis to benzilic acid and ethylpropylaminoethanol. Some N-dealkylation occurred and propylaminoethanol and ethylaminoethanol were excreted in the urine. Benzilic acid and ethylpropylaminoethanol were found in the urine of Parkinsonian patients receiving the unlabelled drug. Whole body autoradiography of mice showed that radioactivity was widely distributed within ten minutes of an oral dose of ^{14}C Benapryzine hydrochloride. The radioactivity in the C.N.S. was found by T.L.C. to be due to unchanged Benapryzine and the presence of Benapryzine in the C.N.S., as shown by the autoradiographs, coincided with the time course of central antiacetylcholine activity of the drug in similar mice. The degree of penetration of Benapryzine into rat brain was studied and evidence was obtained that the unchanged drug penetrated into brain cells. The results were consistent with pharmacological evidence that Benapryzine hydrochloride is a centrally acting antiacetylcholine drug.

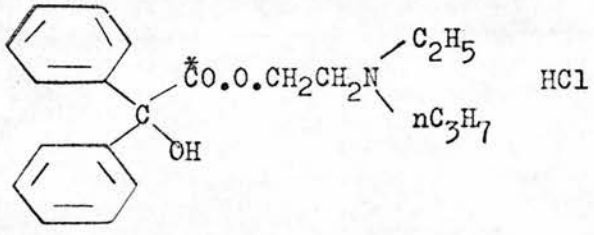
INTRODUCTION

Of a series of basic esters of benzilic acid tested in these laboratories, Benapryzine hydrochloride 2(ethyl-n-propyl-amino)-ethyl-α,α-diphenylglycollate hydrochloride [1] (BRL 1288) was found to have significant central antiacetylcholine activity associated with minimal peripheral activity [2] and it was, therefore, chosen for further pharmacological, toxicological and clinical studies with a view to assessing its potential as an anti-Parkinson drug. The present work on the metabolism and distribution of the drug was undertaken to gain a better understanding of the pharmacological results.

MATERIALS AND METHODS

^{14}C-labelled Benapryzine hydrochloride

Benapryzine hydrochloride labelled with ^{14}C on the carboxyl position was used in most of the experiments.



It was synthesized by the reaction of benzilic acid (carboxyl-^{14}C) with 2-chloroethyl ethylpropylamine in isopropyl alcohol. The labelled benzilic acid was prepared by carbonation with ^{14}C-carbon dioxide (derived from ^{14}C-barium carbonate) of the disodio adduct of benzophenone.

The specific activity of the ^{14}C-Benapryzine hydrochloride was 62.7 μCi/mg. It was found to be radiochemically pure when checked by T.L.C. and autoradiography.

Some of the experiments to determine the route of elimination from the body were performed using ^{14}C-Benapryzine hydrochloride synthesized from benzaldehyde (carbonyl-^{14}C) via benzoin. The drug produced by this route was labelled on the carboxyl carbon and on the α carbon of the α,α diphenylglycollic acid moiety, and had a specific activity of 0.44 μCi/mg.

Animal Studies

Sprague-Dawley rats (Charles River, France) weighing between 150 and 350 g and CF/LP mice (Carworth Europe, Huntingdon, England) weighing 20 g were used.

^{14}C Benapryzine hydrochloride was administered either by gastric intubation or by subcutaneous injection in isotonic saline.

For the excretion and metabolism studies, rats were housed singly in glass metabolism cages designed to effect the separation of urine and faeces. The samples were collected in tubes cooled to -78°. 2-aminoethanol was used to trap any expired ^{14}C-carbon dioxide.

For the study of biliary excretion, bile ducts were cannulated according to the method of Harrison, White and Steward (3) and the rats were kept in close-fitting restraining cages.

In the studies on the distribution of radioactivity, nine mice were dosed orally with ^{14}C-Benapryzine hydrochloride (50 mg/kg. 1 μCi/g). At 5, 10, 20, 40 minutes and 1, 2, 4, 8, 16 and 24 hours after dosing, one of the mice was anaesthetized with ether and killed by immersion in solid carbon dioxide-ethanol. The distribution of radioactivity was investigated by a whole-body autoradiographic technique similar to that used by Ullberg (4). 1 mm sections were cut on a base-sledge microtome in a cold room at -10° and were then exposed at -78° to Kodak Royal Blue X-ray film which was held in wooden cassettes. Control sections from animals which had received no radioactivity were exposed at the same time.

The penetration of radioactivity into rat brain was studied by the method of Nair and Roth (5).

Determination of Radioactivity

Measurements of radioactivity were performed on a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3314) or a Packard Auto Gamma Spectrometer.

The faeces were freeze dried and then weighed portions were oxidized by the method of Dobbs [6].

Urine was diluted to a convenient volume, then 0.5 ml portions were added to 10 ml of Omnifluor (N.E.N. Corp. 4 g dissolved in 1000 ml toluene) and 6 ml of 2-ethoxyethanol. 0.1 ml portions of bile were counted in a similar way.

Blood (0.1 ml) was dissolved in N.C.S. (Amersham/Searle) and bleached with benzoyl peroxide in toluene [7] before the addition of 10 ml of Omnifluor scintillator.

Weighed samples of brain were counted as for blood except that no bleaching was required.

Samples of brain or blood containing ^{131}I were mixed thoroughly with 4 ml of water in plastic tubes before being counted on the Packard Auto Gamma Spectrometer.

Analytical Methods

The radioactive compounds present in whole urine and bile, and faecal or brain homogenates were separated by T.L.C. and located by autoradiography. The biological materials were then acidified to pH 2 with concentrated hydrochloric acid or made alkaline to pH 10 with solid sodium carbonate and extracted with chloroform. The extracts were examined by T.L.C. and autoradiography.

In some experiments, urine or bile samples were mixed with acetate buffer at pH 5.2 and incubated with β-glucuronidase (Sigma) at 37° for 24 hours to hydrolyse any glucuronides present.

Use was made of colour spray-reagents mainly to detect non-radioactive metabolites. Amines were detected with ninhydrin, glucuronides with naphthoresorcinol [8] and benzilic acid and benzilates with concentrated sulphuric acid [9]. Where appropriate, control materials from undosed animals were examined.

T.L.C. was carried out on Silica Gel G prepared according to Stahl (E. Merck, Darmstadt). The solvent systems used are shown in Table 2. Spots on the T.L.C. plates were identified chromatographically by using, as reference compounds, the potential metabolites, which were available as pure compounds.

Human Urine

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24-hour urine samples were obtained from patients with Parkinson's disease who were receiving unlabelled Benapryzine hydrochloride (25 mg t.d.s.). Control urines were obtained from the same patients before the drug was administered.

RESULTS

Excretion Studies

The results of the experiments on rats, summarized in Table 1, show that the excretion pattern was similar irrespective of the route of administration. Over 75% of the radioactivity administered was excreted within 24 hours and the remainder was accounted for within 72 hours.

The table also shows the result of an experiment in which ^{14}C-labelled bile obtained after dosing a rat with ^{14}C-Benapryzine hydrochloride was readministered orally to other rats. An average of 7.3% of the radioactivity was excreted in the urine, demonstrating that some reabsorption occurs.

No radioactivity was detected in the expired air in any experiment.

TABLE 1

Amounts of radioactivity excreted in urine, faeces and bile of rats.

Substance Administered	Route of Admin.	Dose		No. of animals	Interval (hrs.)	Radioactivity Excreted*			
		mg/Kg	µCi			Urine	Faeces	Bile	Total
14C-Benapryzine hydrochloride	p.o.	50	3.6	3	0-24	28 (25-34)	51 (20-68)	-	79 (54-94)
					0-72	31 (26-40)	71 (65-77)	-	102 (99-104)
14C-Benapryzine hydrochloride	s.c.	50	18.5	2	0-24	30 (25,35)	56 (49,63)	-	86 (85,87)
					0-72	32 (26,37)	63 (58,68)	-	95 (95,95)
14C-Benapryzine hydrochloride	p.o.	50	20.0	3	0-12	7 (7-8)	<1	54 (46-67)	61 (53-75)
^{14}C-Bile	p.o.		0.75	3	0-24	7 (6-8)	-	-	-

* The results are expressed as percentages of the dose administered and are given as means with ranges in parentheses.

Identification of Metabolites

Rat Urine:

No unchanged Benapryzine could be detected in urine or extracts of urine. The major radioactive metabolite was identified as benzilic acid. It had the same Rf value as authentic ^{14}C-benzilic acid in each of the solvents used (Table 2) and ran as a single spot when combined with benzilic acid. It gave the same red colour as benzilic acid when aprayed with concentrated sulphuric acid and heated at 100°C for a few minutes. The benzilic acid excreted in the urine accounted for 40% of the total urinary radioactivity.

Other radioactive metabolites appeared to be identical to spots detected with the naphthoresorcinol spray for glucuronides. Incubation of urine with β-glucuronidase increased the amount of radioactivity extractable into chloroform at pH 2 from 40.5% to 61.6% and the radioactive zones which corresponded to glucuronides were no longer detectable.

Three non-radioactive metabolites were detected with ninhydrin and were identified as ethylpropylaminoethanol, propylaminoethanol and ethylaminoethanol by T.L.C., using authentic chemicals as reference compounds.

Human Urine:

Benzilic acid was identified by T.L.C. in a chloroform extract of acidified human urine and ethylpropylaminoethanol with traces of propylaminoethanol and ethylaminoethanol were identified in a chloroform extract of urine which had been made alkaline. No unchanged Benapryzine was detected.

Rat Faeces:

No uncharged Benapryzine was detected in aqueous homogenates of faeces or in chloroform extracts made after the homogenates had been adjusted to pH 10. Benzilic acid was identified in chloroform extracts of acidified homogenates.

Rat Bile:

^{14}C- benzilic acid was found to be present in the bile, together with a number of unidentified radioactive metabolites. As in the case of rat urine, evidence was obtained by T.L.C. that some of the metabolites were present as glucuronides. Incubation of the bile with β-glucuronidase increased the amount of radioactivity extractable into chloroform under acidic conditions from an average of 3.6% to an average of 30.6%. Again, no unchanged Benapryzine was detected.

Table 2 - R_f VALUES OF BENAPRYZINE AND SOME METABOLITES

Substance	Solvent System		
	Butanol 12 Acetic acid 3 Water 5	Hexane 50 Ethanol 150 Ammonia(.880) 3	Butanol 10 Ethanol 10 Ammonia(.880) 1 Water 4
^{14}C Benapryzine	0.56	0.95	0.95
^{14}C-Benzilic acid	0.80	0.38	0.80
Ethylpropylamino-ethanol	0.31	0.51	0.56
Propylaminoethanol	0.37	0.22	0.31
Ethylaminoethanol	0.32	0.13	0.12

The whole-body autoradiographs obtained after dosing mice orally with ^{14}C-Benapryzine hydrochloride showed that the drug was rapidly absorbed and was widely distributed throughout the body within ten minutes of dosing. The drug or its radioactive metabolites had penetrated into the C.N.S. by this time and high concentrations of radioactivity were found in the stomach, intestine, liver, kidney, urine and Harder's gland. At forty minutes, the generally high level of radioactivity was beginning to decrease and, eventually, by four hours radioactivity was localized mainly in the liver, gall bladder, intestine and urine. Radioactivity was practically absent from the C.N.S. by two hours. At twenty-four hours, very little radioactivity remained and was localized in the liver and intestine.

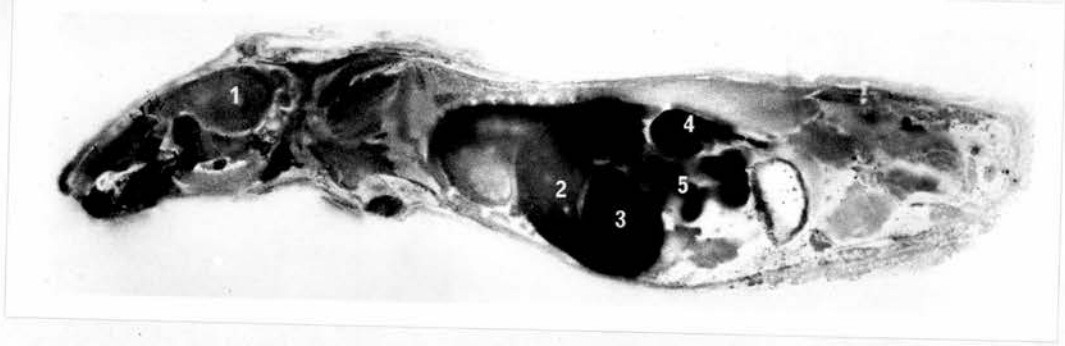
In the accompanying photographs of the whole-body autoradiographs, a higher intensity of black indicates a higher concentration of radioactivity.

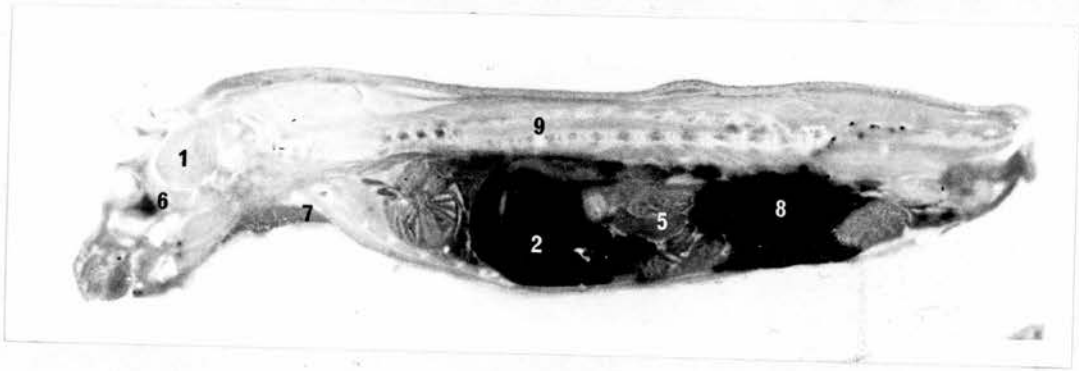
Fig.1 Distribution of radioactivity in mice, 10 mins (A) 1 hour (B), and 4 hours (C) after oral administration of ^{14}C-Benapryzine hydrochloride

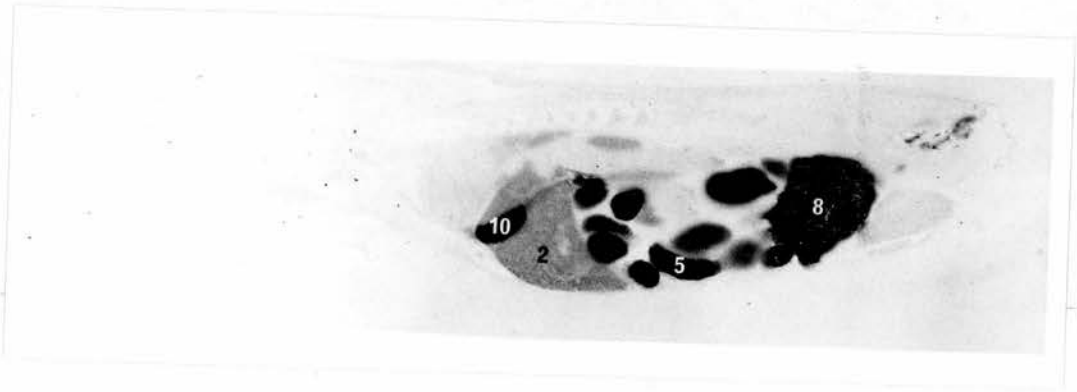
1 = Brain 2 = Liver 3 = Stomach 4 = Kidney

5 = Intestine 6 = Harder's gland 7 = Salivary gland

8 = Urine 9 = Spinal cord 10 = Gall bladder







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1 = Brain 2 = Liver 3 = Stomach 4 = Kidney

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Brain penetration studies

Extracts of mouse brain made at 10, 20, and 30 minutes after oral or sub-cutaneous dosing with ^{14}C-Benapryzine hydrochloride were examined by T.L.C. and autoradiography and were found to contain unchanged drug. After oral dosing, three unidentified metabolites were also present.

The results of an experiment to determine the degree of penetration of radioactivity into rat brain are given in Table 3. The relative concentrations of radioactivity in each of the regions of brain studied were higher after intravenous administration of ^{14}C-Benapryzine hydrochloride than after ^{14}C-urea, ^{35}S-sulphate or ^{131}I-serum albumen.

The radioactivity in rat brain, 30 minutes after an intravenous dose of ^{14}C-Benapryzine was found by T.L.C. and autoradiography to be due to unchanged drug.

Relative concentrations of compounds in rat brain after i.v. injection of Benapryzine or reference compounds

Tissue	Relative concentration of compounds in rat brain			
	^{131}I-serum albumen	^{35}S-sulphate	^{14}C-urea	^{14}C-Benapryzine
Olfactory lobes	0.230 ± 0.020	7.12 ± 1.78 (5)	56.3 ± 3.6	119.0 ± 6.8
Cerebral cortex	0.284 ± 0.013	5.35 ± 0.50	44.5 ± 2.2	173.1 ± 19.0
Caudate Nucleus	0.180 ± 0.009	2.73 ± 0.14	39.8 ± 1.7	155.7 ± 14.0 (5)
Hippocampus	0.232 ± 0.016	3.47 ± 0.48	43.4 ± 1.4	162.3 ± 14.2
Thalamus + Hypothalamus	0.349 ± 0.025	5.20 ± 0.42	41.3 ± 2.0	141.0 ± 16.3
Cerebellum	0.353 ± 0.016	6.20 ± 0.67	44.3 ± 1.9	115.4 ± 9.3
Medulla and spinal cord	0.171 ± 0.006	3.32 ± 0.29 (5)	44.0 ± 2.1	143.7 ± 11.3
Dose (μCi/kg)	245	500	100	100
Interval between injection and killing (mins)	5	5	60	30

The data are expressed as relative concentrations i.e. $\frac{\text{Amount (d.p.m.) per mg of brain (wet weight)}}{\text{Amount (d.p.m.) per μl of blood}} \times 100$ and are given as means ± standard errors. The number of animals in each group was six except where stated.

The principal route of excretion of radioactivity after administration of ^{14}C-Benapryzine hydrochloride to rats is via the bile. The amount of radioactivity recovered in the bile in 12 hours after oral administration is about the same as the amount excreted in 24 hours in the faeces of intact animals, indicating that the drug is well absorbed from the gut after an oral dose.

Benapryzine hydrochloride is extensively metabolized by rats and the excretion of unchanged drug was not detected. The major route of metabolism is hydrolysis of the ester bond to give benzilic acid and ethylpropylaminoethanol. Evidence was obtained that some acidic metabolites are excreted in the urine and bile as glucuronide conjugates. Some dealkylation of the drug also occurs giving rise to ethylaminoethanol and propylaminoethanol. It is not clear whether the intact ester undergoes dealkylation before hydrolysis, or whether it is first hydrolysed to give ethylpropylaminoethanol which is subsequently dealkylated. Benactyzine, which differs from Benapryzine only by the presence of an ethyl group in place of the n-propyl group, can be dealkylated before being hydrolysed. Edelson and Douglas (10) have shown in rats following intraperitoneal injection of Benactyzine that one of the metabolites excreted in the urine is the N-de-alkylated product, ethylaminoethyl benzilate. However, neither of the N-dealkylated products of Benapryzine was detected in the urine after oral dosing, the limit of detection being about 1% of the dose.

From the preliminary studies carried out, Benapryzine hydrochloride is metabolized in man in the same way as in rats, the principal urinary metabolites being identical in both species.

The whole body autoradiographs of mice showed that, in this species, like rat, the bile is an important route of excretion of radioactivity, for the gall-bladder contained prominent concentrations of radioactivity for up to 8 hours after dosing. The persistence of radioactivity in the liver and intestine at 24 hours after dosing is probably due to enterohepatic circulation of pharmacologically inactive metabolites.

Benapryzine is believed to act on central cholinergic mechanisms since it inhibits the tremors induced by oxotremorine in mice [2, 11]. The duration of pharmacological activity found in this test * coincides with the duration of radioactivity in the central nervous system as demonstrated by autoradiography. The radioactivity in the central nervous system was shown to be due to unchanged ^{14}C-Benapryzine, therefore it is probable that the unchanged drug is responsible for the central pharmacological activity.

Using the technique described by Nair and Roth [5], evidence was obtained that Benapryzine penetrates into the brain cells of rats. The relative brain to blood concentrations of Benapryzine in each of the regions of brain studied, were found to be higher than those of ^{14}C-urea which was used as an example of a compound capable of penetrating into cells. The drug was not found to be concentrated in any specific region of the brain.

The results presented in this paper support the pharmacological evidence [2, 11] that Benapryzine hydrochloride is active in Parkinson's disease through its action in the central nervous system.

* R. O. Hughes, personal communication

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ASSAY OF ATROPINE-LIKE DRUGS FOR INHIBITION OF GASTRIC SECRETION IN THE PYLORIC LIGATED (SHAY) RAT

BY

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(Received for publication 8-10-1962*).*

The Shay rat (SHAY, KOMAROV, FELS, MERANZE, GRUENSTEIN, and SIPLET, 1945) can be used to estimate the effect of atropine-like drugs on the gastro-intestinal tract. Some of the methods depend on the prevention of rumenal ulceration which occurs within 24 hours following pyloric ligation (BARRETT, RUTLEDGE, PLUMMER and YONKMAN, 1953; CAHEN and TVEDE, 1951; KOWALEWSKI, MACKENZIE, SHNITKA and BAIN, 1954; MÜNCHOW, 1954). Other workers (GYERMEK and NÁDOR, 1957; VAN PROOSDIJ-HARTZEMA, JANSSEN and DE JONGH, 1955; SHEA, 1956; VISSCHER, SEAY, TAZELAAR, VELDKAMP and VANDER-BROOK, 1954) have used the inhibition of the volume of gastric juice secreted by the 2–8 hour preparations as an index of activity of the drugs. No quantitative assessment of the amount of acid secreted has been made in any of these studies, change in acidity usually being expressed simply as an alteration in pH. Since none of the methods used has given a precise estimate of potency in relation to a standard drug the present study was therefore carried out to evolve a more reliable assay procedure.

METHOD

Albino rats weighing 100–200 g, of either sex were starved for 24 hours in cages with wide mesh bottoms in order to prevent coprophagy. Four or 5 rats were maintained in each cage. Water was allowed ad lib.

The rats were anaesthetized with ether and after laparotomy the duodenum was ligated with nylon thread about 0.5 cm below the pyloric sphincter. The abdominal muscle layers were sutured and the skin closed with Michel clips. 4.0 ml. of warm 0.9 % saline were given intraperitoneally. The drugs were dissolved in 0.9 % saline and 0.1 ml./100 g body weight was administered subcutaneously. After 2 or 6 hours, according to the conditions of the test, the rats were again anaesthetized and the abdomen opened. The oesophagus was ligated just below the diaphragm, the stomach removed, and the animal killed. After removal of the adhering mesentery from the stomach its contents were drained into a centrifuge tube through a slit in the greater curvature. After centrifuging for 10–15 min., at 2,000 r.p.m., the total volume of the juice was measured, due allowance being made for the solid content. The free acidity was estimated by taking 1.0 ml. of the supernatant and titrating with 0.01 N.NaOH, 6 drops of Topfers reagent being used as indicator. If the volume of juice recovered from one rat in a group was less than 1.0 ml. all the samples from that group were pooled.

At various intervals after pyloric ligation the degree of pupil dilatation was also estimated and scored as follows:

75 — 100 %maximum dilatation 4
50 — 75 % dilatation 3
25 — 50 % dilatation 2
5 — 25 % dilatation 1

Drugs used were atropine sulphate, atropine methylnitrate, poldine methylsulphate (Nacton), and oxyphenonium bromide (Antrenyl).

Results

Effect of Sex and Weight

The effect of sex and weight of the rats on the volume and acidity of the juice secreted was ascertained from all the 2 and 6 hour untreated control rats. The animals were divided into groups of increasing weight range, and the volume of the juice and free acidity measured. The results are given in Table I and an analysis of variance showed that in the various weight ranges examined there was no significant difference in the volume of juice nor in the concentration of free acid secreted. There was also no significant differences in the response between the sexes.

Correlation between volume of secretion and free acid concentration

To assay atropine-like drugs it is necessary to establish that the

TABLE I

Volume (ml) *and free acid concentration* (mEq./litre) *in untreated, control 6 and 2 hour pyloric ligated rats. Mean values with standard error of mean for n* (number of rats) *in each weight range*

Period of Ligation	Sex	Gastric Juice	Weight Range 101–120 g		121–140 g		141–160 g		161–180 g		181–200 g	
			Mean ± S.E.	*n*	Mean ± S.E.	*n*	Mean ± S.E.	*n*	Mean ± S.E.	*n*	Mean ± S.E.	*n*
6 hours	♂	Volume ml	6.3 ± 1.2	8	7.0 ± 0.4	39	7.0 ± 0.3	43	6.7 ± 0.7	19	6.9 ± 0.6	10
		Free Acid Conc.	89.6 ± 8.4	8	100.7 ± 2.8	39	94.8 ± 2.2	43	93.3 ± 3.6	19	90.6 ± 4.8	10
	♀	Volume ml	6.8 ± 0.7	10	6.9 ± 0.4	46	8.0 ± 0.4	36	8.5 ± 1.0	8		
		Free Acid Conc.	98.1 ± 7.2	10	94.2 ± 3.7	46	101.9 ± 1.9	36	97.6 ± 5.3	8		
2 hours	♂	Volume ml	3.2 ± 1.2	2	2.9 ± 0.4	10	3.1 ± 0.4	9	3.2 ± 0.6	5		
		Free Acid Conc.	73.5 ± 9.1	2	71.9 ± 11.8	10	76.0 ± 10.3	9	78.4 ± 6.1	5		
	♀	Volume ml	2.3 ± 0.2	36	2.7 ± 0.4	16	2.4 ± 0.4	5				
		Free Acid Conc.	65.0 ± 5.2	36	72.7 ± 9.0	16	67.6 ± 13.5	5				

TABLE II

Dose response line for atropine sulphate in the 6 hour pyloric ligated rat

	DOSE OF ATROPINE SULPHATE									
	6.25 mg/kg		1.25 mg/kg		0.25 mg/kg		0.05 mg/kg		Control	
	x	*y*	*x*	*y*	*x*	*y*	*x*	*y*	*x*	*y*
	0.8	0	2.0	68	3.6	37	4.7	67	4.9	99
	2.3	60	1.5	55	4.2	78	2.8	79	5.3	107
	0.7	0	3.6	50	2.7	121	2.8	53	6.8	62
	1.7	50	3.6	54	5.8	100	4.4	74	3.5	79
	0.9	0	1.7	64	1.4	48	3.8	88	7.6	101
	1.4	45	1.1	39	4.0	64	4.1	60	7.1	74
	2.1	47	1.1	39	5.1	78	4.0	38	6.6	72
	1.7	49	2.8	59	4.8	38	7.1	70	7.3	70
	1.0	17	1.4	46	4.4	60	2.3	110	9.2	80
	1.2	28	2.8	71	2.9	43	7.5	75	5.1	67
	3.7	71	3.7	73	5.2	44	6.5	61	5.9	75
	1.3	13	3.1	33	3.5	69	6.9	71	12.4	64
	0.3	97	1.8	50	1.9	76	4.2	100	9.0	81
	1.6	55	3.3	41	3.3	44	4.0	90	3.3	51
	0.8	97	1.9	101	6.2	99	2.2	33	3.8	85
	1.7	41	4.7	56	5.2	52	1.7	0	7.6	77
	0.4	30	4.7	77	3.3	67	3.5	56	6.4	54
	0.4	30	2.4	45	2.7	43	6.3	89	9.0	70
	0.4	30	4.6	66	1.7	89	3.5	57	6.7	53
	0.3	43	2.6	54	3.8	49	4.6	69	3.2	56
Total	24.7	803	54.4	1,141	75.7	1,299	86.9	1,340	130.7	1,477
Mean	1.2	40	2.7	57	3.8	65	4.3	67	6.5	74

ANALYSIS OF COVARIANCE

Source of Variation	Degrees of Freedom	Sum of Squares and Products			Errors of Estimate		
		Sx^2	Sxy	Sy^2	Sum of Sq**	Degree of Freedom	Mean Sq.
Level	79	241.74	149.38	510.37	418.07	78	
Doses	3	112.34	89.36	98.79			
Within Rats	76	129.40	60.02	411.58	356.91	75	4.76
For test of significance of adjusted means					61.16	3	20.39

x — Volume of gastric juice (ml). *y* — Free acid concentration (mEq HCl/litre).

$$F = \frac{20.39}{4.76} = 4.28 - (P > 0.05 < 0.01)$$

$$* \; Sy^2 - \frac{(Sxy)^2}{Sx^2}$$

relationship between log dose and the reduction in volume and acidity of the gastric juice is linear. Four doses of atropine sulphate, 0.05, 0.25, 1.25 and 6.25 mg/kg were administered to groups of 4 rats which were set up simultaneously with a control group. The procedure was repeated on successive days until a total of 20 rats had been employed at each dose level.

The values for volume and free acid concentration of gastric juice are given in Table II. The relationship between free acid concentration and volume of gastric juice was examined by co-variance analysis, (SNEDECOR, 1946), and it was shown that a significant correlation ($F = 4.28$) between the 2 variables existed. In all determinations of the effect of drugs on gastric secretion we therefore measured only the volume of juice secreted by each animal. This was then converted to a percentage of the mean volume of juice secreted by the control group for each day of the test.

Assays

A number of 2 × 2 assays were performed with known anti-acetylcholine drugs. Atropine sulphate was used as the standard. A high dose and low dose of the compound was selected so that the high dose would give an inhibition in the volume of juice secreted of about 70 to 80 % and the low dose about 20 % of the control volume. A dose ratio of 1 : 12 for all compounds tested was found to be satisfactory. Five rats were used at each dose level for each day of the test together with a control group, untreated, of 5 rats. The test was repeated every day for 4 days so that 20 rats were used for each dose. The percentage inhibition of the volume of juice was calculated as previously and the results of the 4 tests were pooled. The results were analysed by the method of SCHILD (1942) and the relative potency with 5 % limits of error calculated.

In order to obtain some measure of the duration of action the pupil diameters were scored at 2, 3, 4 and 6 hours after dosing in the 6 hour test, and at 2 hours only in the 2 hour test.

The assay results are given in Table III and the relative effects on gastric secretion and mydriatic effects are shown in Fig. 1. In none of the assays was there a significant deviation from parallelism ($P > 0.1$) and the variance due to regression was always highly significant ($P < 0.001$). In relation to atropine sulphate, atropine methylnitrate and oxyphenonium were respectively 1.7 and 5.2 times more active in the 6 hour than in the 2 hour Shay rat. On the other hand, poldine methylsulphate has the same activity in both the 6 and 2 hour preparation.

The mydriatic response due to atropine methylnitrate and poldine methylsulphate in both the 2 and 6 hour preparations parallels the activity of atropine sulphate but the effect of oxyphenonium is more prolonged than that of atropine sulphate. This effect is more noticeable at the lower dose levels.

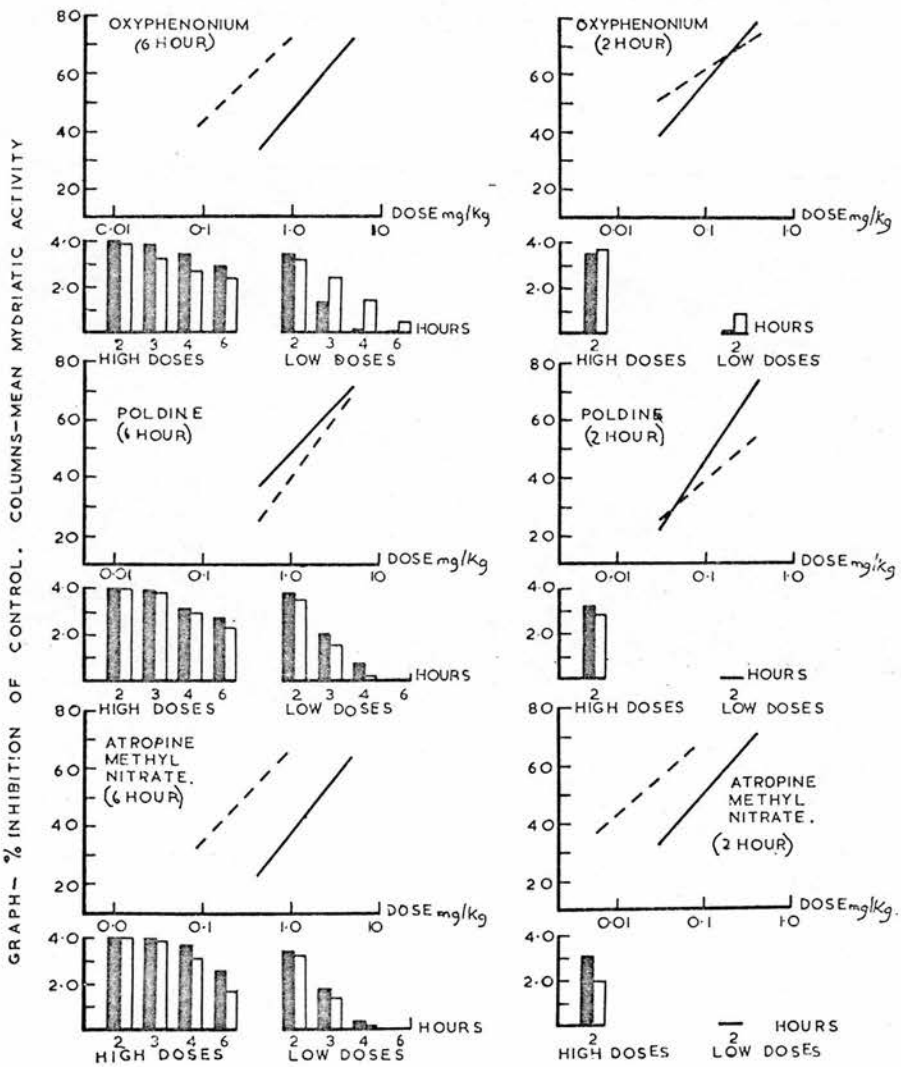

FIG. 1

Assays of atropine-like drugs in the 2 and 6 hour pyloric ligated rat. Continuous dose response lines — atropine sulphate and broken line — drugs. Mydriatic activity, mean score for pupil diameter of twenty rats, black columns — atropine sulphate, blank columns — drug.

TABLE III

Relative potencies of atropine-like drugs in terms of atropine sulphate as evaluated by the inhibition of volume of gastric juice in the 2 and 6 hour pyloric ligated rat

$$\lambda = \frac{\sqrt{\text{Mean Square Error}}}{\text{Slope}}$$

Compound	Relative Potency with 5 % limits of error (P = 0.95). Atropine sulphate = 1.0			
	6 hour	λ	2 hour	λ
Atropine Methyl Nitrate	7.9 (4.0–15.4)	0.50	4.7 (2.0–10.9)	0.69
Oxyphenonium	7.3 (3.4–15.5)	0.56	1.4 (0.7–2.6)	0.60
Poldine	0.6 (0.2–1.2)	0.63	0.6 (0.4–1.1)	0.55

DISCUSSION

In the 4 hour pyloric ligated rat, SHAY, SUN and GRUENSTEIN (1954), found that although there was no significant difference between males and females, there was an increase in the rate of secretion and acid output with increase in weight. In the present study with 2 and 6 hour pyloric ligated rat it was found that over the weight range used (100–200 g) neither sex nor weight had any significant effect on the volume of secretion. In the assays therefore, no adjustment for weight was made in expressing the volume of secretion.

A 24 hour period of starvation was considered adequate, as in the majority of cases the volume of solid present in samples of gastric juice after pyloric ligation was 0.2 ml. or less. Such a period of starvation did not appear to have any detrimental effect on the general condition of the animals. Other workers (CAHEN and TVEDE, 1951) have used up to 72 hours as the period of starvation, but according to MADDEN, RAMSBURG and HUNDLEY, (1951) a 72 hour starvation can influence the rate of secretion and causes a lowering of resistance of the mucosa with consequent haemorrhagic erosions and buffering of gastric juice.

In our experiments there was considerable variation in the volume of juice secreted in the untreated control rats. The mean volume secreted by a group of 219 rats was 7.1 $\pm$ 2.2 ml. (S.D.) in 6 hours and 2.6 $\pm$ 1.2 ml. (S.D.) in a groups of 83 rats in the 2 hours. In view of this we therefore decided to use a minimum of 20 rats at each dose level for studying the inhibitory effect of atropine-like drugs on gastric secretion.

For assay purposes it is necesary to set up a control group simultaneously with the groups under test. The activity at a dose level is then expressed as a percentage inhibition of juice secreted by the control group instead of simply measuring the depression in volume secreted between the groups without reference to the control. The former procedure takes into account solely the effect of the drug on the secretion which can be inhibited by atropine-like drugs and is not complicated by any basal secretion which can vary to an unknown degree.

Rats used in the 6 hour assays gave mydriatic responses indicating that the drugs differed in their duration of action. Under the circumstances, therefore, the relative effect of the drug may vary according to the time at which the animals are killed. This was confirmed in the 2 hour preparation. Atropine methylnitrate and oxyphenonium in the 6 hour preparation were 7.9 and 7.3 times as active as atropine sulphate respectively, while in the 2 hour preparation the activities were 4.7 and 1.4 respectively. On the other hand, poldine methylsulphate had the same relative activity at both time intervals, indicating that its duration of action was similar to atropine sulphate. In quoting the relative activities of atropine-like drugs *in vivo* the time at which the final reading is made should always be stated. In addition, the *in vitro* anti-acetylcholine activity of the drugs should also be given in order to indicate the absolute activity of the compound; this absolute value being indeterminable in the Shay rat preparation.

The assay procedure as described gives reproducible results and relative activities can be quoted together with fiducial limits. The index of precision (λ) does not indicate a high degree of accuracy but in all the assays carried out it is consistent.

Summary

An assay procedure using the inhibition of volume of gastric juice in the 2 and 6 hour pyloric ligated rat is described. Relative potencies of oxyphenonium, poldine methylsulphate and atropine methylnitrate in terms of atropine sulphate are given for both 2 and 6 hour preparations. The importance of the duration of action of the drug under test in comparison with that of the standard is emphasized.

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INTRODUCTORY PAPERS

PRACTICAL ASPECTS OF STRAIN VARIATION IN RELATION TO PHARMACOLOGICAL TESTING

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THE control of variation in animal experimentation particularly in the field of bioassay and pharmacological screening is perhaps one of the principal problems confronting the industrial pharmacologist. The application of statistical procedures and design to problems of toxicity and bioassay by Trevan (1927) and Gaddum (1933) pointed the way to refinements in experimental method thereby improving the accuracy of the tests. Further advances in bioassay procedure resulted from the classical research of Fisher who used statistical methods to determine precisely the variation due to different factors within the experiment and then by appropriate design, considering each factor, limited the variation to a minimum. With a view to reducing variation still further a number of industrial laboratories concentrated on the production of inbred lines in the hope that more constant and uniform responses would be obtained. This largely became established practice although little experimental evidence was brought forward to support the belief. Mather (1946) subsequently observed that F_1 hybrids gave more uniform responses and were generally more vigorous than either of the parent inbred strains. These observations were largely overlooked until McLaren and Michie (1954) and Biggers and Claringbold (1954) independently re-discovered this phenomenon. Biggers, McLaren and Michie (1961) feel in general that the F_1 hybrid is the most satisfactory laboratory animal. Chai (1960) has put forward an opposing viewpoint stating that "For the assay of a given substance, the choice of assay animal—either inbred or an F_1 hybrid—cannot be made in advance; it has to be based on existing data or determined experimentally". Brown (1961a, b) has lent additional experimental evidence to support this.

Environmental conditions, however, also play a vital part in biological assay. Chance has carried out extensive investigations into the effects of altering the environmental conditions. These investigations have been admirably summarised by Russell and Burch (1959).

Finally in this respect mention must be made of the work of the Laboratory Animals Centre under the direction of Dr. Lane-Petter and of the Animal Technicians Association who have done so much to improve the general quality and health of the laboratory animals. Without a healthy robust animal all experimental work involving animals, whether pharmacological or bacteriological, can be rendered virtually worthless.

Differences in the physiological response of various strains of mice have been clearly recognised. Elizabeth Russell (1955) in an excellent review of the "Significance of Physiological Pattern of Animal Strains in

Biological Research", has drawn attention to the considerable differences that occur between mice to disease susceptibility, nature of disease produced by a given pathogen, survival time of infected individuals; capacity for antibody production, cold tolerance, reaction to specific toxins; sensitivity to and content of various hormones and reaction to endocrine extirpation; differences in normal blood-cell levels, life-expectancy and pathological pattern. The pharmacologist has been perhaps rather slower to give consideration to strain variation and it is the purpose of this paper to draw attention to the considerable variations which may exist and to indicate how, by correct selection of strain the quality of pharmacological assays may be improved.

Siegmund, Cadmus and Lu (1957) have developed a screening technique for mild analgesic drugs which has gained considerable acceptance among industrial pharmacologists. In this test phenylquinone is injected intraperitoneally to mice and a typical response is produced which can be inhibited by analgesic drugs. Phenylquinone produces a characteristically biphasic response in a number of strains of mice—intermittent contraction of the abdomen, rubbing the abdomen on the floor of the cage and stretching of the hind limbs. Unless an adequate degree of this writhing response is produced, the test gives erratic and non-reproducible results (Hendershot and Forsaith, 1959). Similarly in our own laboratory we were unable to get satisfactory responses and we therefore decided to examine as many strains as possible in order to select the one which gave best writhing response. We obtained eight strains through the courtesy of Dr. Lane-Petter of the Laboratory Animals Centre, Carshalton, and three other strains from commercial sources all of which were examined for their writhing response.

Methods

Random samples of 6 male mice, 5 to 7 weeks old, from each strain were injected intraperitoneally with 2 mg./kg. phenylquinone and the individual reaction of each mouse was recorded for a period of 30 min., noting the cumulative number of writhes at 2 min. intervals. Two mice from each strain were tested on each of 3 days, the strains being randomised throughout the test days. From these results the frequency of writhing during each 2 min. period was computed. The phenylquinone was injected as an 0·2 mg./ml. solution in 5 per cent ethanol and was maintained at 37°, the solution being just below saturation at this temperature. In all tests the technician was unaware of the strain being used. In tests where aspirin was administered it was given orally 50 min. before the phenylquinone.

Strains used:

Pure inbred strains		Random-bred albino strains (nominal designation)
CBA	A2G	
C57BL/6	C57L	ALB1
DBA/2	C_3H	ALB2
C57Br/cd	CE	ALB3

Results

The frequency of writhing in eleven strains of mice is illustrated in Fig. 1, and the total number of writhes per mouse in a 30 min. period following administration of the phenylquinone is shown in Table I. The mice can be divided into three groups: those which show a large number of writhes

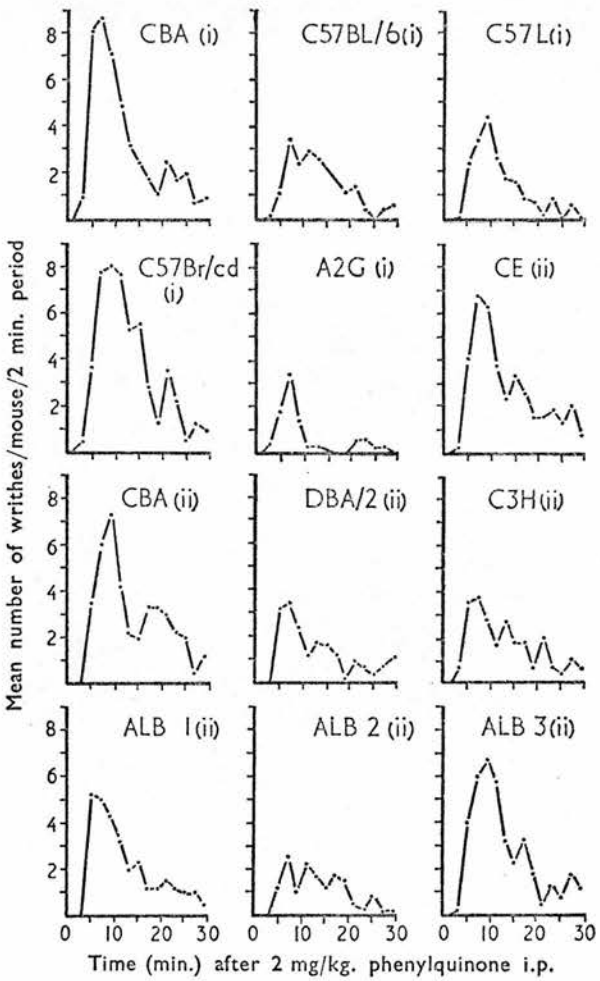

Fig. 1. Frequency of writhing of 11 strains of mice.

CBA, C57Br/cd, CE and ALB3; those which are intermediate in responses C57BL/6, C57L, C_3H and ALB1, and those which give poor responses A2G, ALB2 and DBA/2. The characteristics of the writhes also differ between those groups. In the strain which give a large number of writhes, the syndrome is quite distinct but in the others the phases are less clearly seen, and in the poorly responding animals a writhe may only be detected as a slight contraction of the abdomen or stretch of the limbs.

TABLE I

NUMBER OF WRITHES PER MOUSE IN 30 MIN. FOR EIGHT INBRED AND THREE COMMERCIAL MOUSE STRAINS FOLLOWING INTRAPERITONEAL ADMINISTRATION OF 2 MG./KG. PHENYLQUINONE

Experiment	Strain	Weight range (g.)	Writhes per mouse						Mean
			No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	
(i)	CBA	14–17	38	60	16	90	41	38	47
	C57BL/6	12–15	3	16	49	19	8	22	19
	C57L	21–25	22	21	32	20	1	30	21
	C57Br/cd	15–18	47	17	53	65	49	75	51
	A2G	15–18	8	16	9	9	0	10	9
(ii)	CE	14–19	36	39	0	49	75	34	39
	CE*	13–15	43	40	34	25	33		35
	CBA	21–24	24	54	34	28	65	39	41
	DBA/2	8–14	7	1	20	36	43	14	20
	C_3H	20–23	12	27	55	20	10	21	24
	ALB1	20–23	40	62	5	25	13	32	29
	ALB2	17–19	17	12	7	18	16	19	15
	ALB3	15–19	39	18	33	77	43	24	39

* Litter mates.

Individual variation in the number of writhes within groups is also high even in mice which respond well but this does not detract from using the phenylquinone test to give a graded response with analgesic drugs. Records of the writhing frequencies of batches of five mice from one strain (ALB3) show that 15 mice will give a mean number of writhes of 129 $\pm$ 11·0 S.E.M. For a more accurate test the use of litter mates would obviously reduce this error still further (Table II) but this would generally be impracticable in a screening programme.

TABLE II

MEAN NUMBER OF WRITHES AND A COMPARISON OF THE COEFFICIENTS OF VARIABILITY AT 20 MIN. AND 30 MIN. IN RESPONSE TO INTRAPERITONEAL ADMINISTRATION OF 2 MG./KG. PHENYLQUINONE, IN DIFFERENT MOUSE STRAINS

Experiment	Strain	20 min.		30 min.	
		Mean no. of writhes	Coefficient of variation	Mean no. of writhes	Coefficient of variation
(i)	CBA	39·0	51	47·2	53
	C57BL/6	16·3	85	19·5	83
	C57L	18·7	58	21·0	55
	C57Br/cd	42·7	42	51·0	39
	A2G	7·3	54	8·7	59
(ii)	CE	31·7	62	38·8	63
	CE*	28·8	22	35·0	20
	CBA	31·8	38	40·7	39
	DBA/2	15·8	79	20·2	81
	C_3H	19·3	65	24·2	68
	ALB1	24·3	73	29·5	69
	ALB2	12·8	34	14·8	31
	ALB3	33·3	51	39·0	54

* Litter mates.

The phase of most frequent writhing is complete in 20 min. although the mice continue to writhe for some time afterwards but at a much reduced rate. No significant difference was found between the estimates of the variability of the number of writhes in 20 min. and 30 min. periods, therefore it is unnecessary to count for more than 20 min.

In order to verify that mice obtained from one source would still retain their writhing characteristics even when reared in a different laboratory, breeding pairs of CBA and C57BL/6 were obtained from the Laboratory Animals Centre. The first strain gave a good writhing response and the second an intermediate response. We raised sufficient numbers to repeat the tests, the results of which are shown in Fig. 2. The curves of the second test overlap precisely the initial curves obtained with both strains.

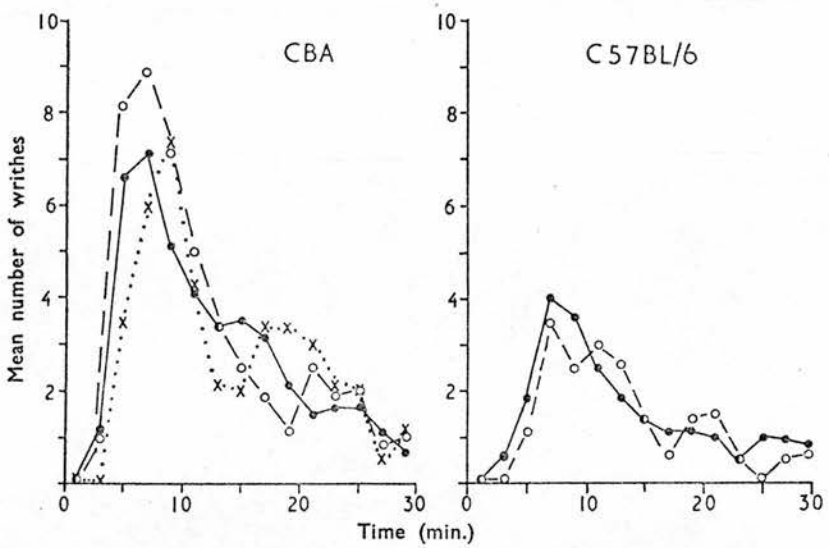

FIG. 2. Frequency of writhing of two strains of mice ○ – – ○ March, 1961 (6 mice); × – – × April, 1961 (6 mice); ●—● November, 1961 (30 mice).

Finally having shown that a mouse strain writhes consistently and then having selected a strain which gives an adequate degree of writhing, it is also necessary to confirm that the animals continue to give the same response to the analgesic drugs (Fig. 3). The dose response line obtained

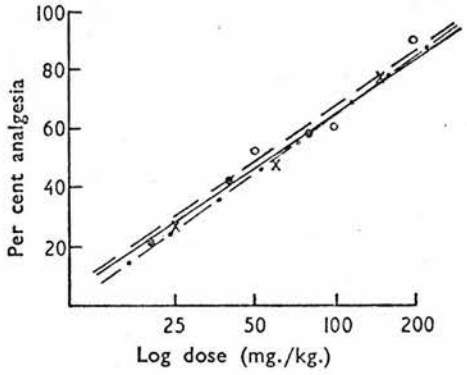

FIG. 3. Single day analgesic assays of aspirin, 15 mice/dose. ●—● 9.12.1960; ○ – – ○ 6.12.1961; × – – × 11.12.1961.

on 9/12/60 for the mean writhing response to graded doses of aspirin for the 20 min. period following phenylquinone was compared with the line obtained exactly a year later (6/12/61) using the same strain of mouse. The two lines were found to be in very close alignment. A further dose response line prepared 5 days after the last test (11/12/61) gave a slope which was also virtually identical to the other two. The ED50 values for each test were respectively 58, 54 and 60 mg./kg. Mice which responded poorly to the phenylquinone failed to give a linear response to aspirin.

Discussion

By consideration of the genetical, physiological and environmental background of the strain of animal used in assay and screening procedures the quality of a test can be considerably improved with a corresponding reduction in error variance. The sensitivity and accuracy of tests can be enhanced further by the selection of the strain of animal which gives the best pharmacological response. It is not uncommon that many pharmacological testing procedures are acceptable to one laboratory and not to another. The reason for these discrepancies can largely be found in the strains of animal used in the different laboratories. The results with phenylquinone writhing response indicate that there is a large variation between mouse strains, probably much larger than hitherto suspected, and that some strains give such poor responses that it is impossible to get a quantitative response to mild analgesic drugs. The expediency of using a selected strain which gives a high degree of writhing in the phenylquinone test is demonstrated by the consistency in the dose response lines obtained with aspirin and in the comparatively low doses of mild analgesic drugs required to achieve a pronounced and consistent reduction in the frequency of writhing. Additional support to this is provided by Dr. P. F. D'Arcy who tells us that, by using a similar strain of mouse and employing identical assay procedure, he has obtained ED50 values, mean 52 mg./kg. for aspirin. These figures which were obtained independently agree remarkably with our own.

Whilst it is not generally practicable to screen a wide selection of strains before performing every pharmacological test, this would be advantageous when establishing a particular long term programme for the screen or assay of a particular series of drugs. In any pharmacology laboratory, it would be useful to have available two to three strains of mouse of widely differing characteristics (possibly one or two home strains supplemented by commerical stocks), so that should any test appear not to be satisfactory, although other workers have found it to be so, the test could be performed in different strains. Certainly we would advocate that coloured mice be more widely used in pharmacology as well as albino mice instead of the present almost exclusive use of the latter.

With some species, the choice of strain is naturally more limited than with the mouse and it would not be practicable, let alone economical, to screen a variety of strains. Nevertheless, strain variation should be borne in mind as of practical importance to an experiment rather than simply theoretical, particularly when wide discrepancies of results appear between

work performed in different laboratories. It should not, however, become the scapegoat for all unsatisfactory results.

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SHORT-TERM STUDY OF THE EFFECT OF PHENACETIN, PHENAZONE AND AMIDOPYRINE ON THE RAT KIDNEY

BY

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SHORT-TERM STUDY OF THE EFFECT OF PHENACETIN, PHENAZONE AND AMIDOPYRINE ON THE RAT KIDNEY

BY

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(*Received April* 12, 1967)

Phenacetin has been implicated in kidney damage in man (Spuhler & Zollinger, 1953); however, the number of cases of kidney damage from phenacetin consumption reported from Switzerland, Germany and Scandinavia is greater than that reported from Australia and the United States, though the average consumption of phenacetin per head is similar in all these countries (Ross, 1962). The reported incidence of "analgesic nephritis" is also low in Great Britain, where the consumption of phenacetin is not less than in the other countries (Prescott, 1965).

In preparations examined by Horisberger, Grandjean & Lanz (1958) and Grimlund (1965) the drugs most frequently administered with phenacetin were phenazone and amidopyrine or a close analogue. We felt that any of these drugs could be responsible for the renal effects and we have therefore carried out a preliminary investigation in rats comparing the toxicity of phenacetin, phenazone and amidopyrine, with particular reference to effects on the kidney.

METHODS

Phenacetin, phenazone and amidopyrine were administered orally on 7 days a week as a suspension in 1.25% w/v methyl cellulose in water to young, mature Sprague-Dawley strain male rats (Charles Rivers, U.S.A.), initial weight range, 200–220 g, according to the dosage schedule in Table 1.

Urine cytology and routine urine analysis

Not less than four animals from each group were given an oral water load equivalent to 3% of their body weight. Immediately following this any urine in the bladder was expressed by suprapubic pressure. The cells and particulate material in a measured aliquot of the urine collected during the following 2-hr period were then concentrated fivefold by centrifuging for 5 min at 2,000 r.p.m., with removal of the appropriate quantity of the supernatant fluid. A portion of the cell concentrate was then stained with a peroxidase stain (Prescott & Brodie, 1964), before quantitative assessment in a Fuchs–Rosenthal counting chamber. At this stage the numbers of squamous cells, peroxidase-positive cells, erythrocytes, and cells of probable renal origin, were recorded. After centrifuging, some of the deposit was transferred to albuminized slides, fixed in alcohol, and stained with EA 50 polychrome Papanicolaou stain (Ortho Pharmaceuticals Limited). The provisional morphological classification made in the counting chamber wet preparation was verified using these films.

The remainder of the urine was examined for protein and sugar using Haemocombistix (Ames Division of Miles Laboratories Limited). The amount of protein required to produce an arbitrary series of colour changes on the chemically treated strip was assesesed previously by assaying a series of standards on the Autoanalyser, using both the biuret and a turbidometric method. Thus, a ± reaction is approximately equivalent to a urinary protein of less than 20 mg/100 ml., a + of 20–40 mg/100 ml., a ++ of 40–60 mg/100 ml., and a +++ of not less than 60 mg/100 ml.

Finally, urinary glutamic oxalacetic transaminase was determined on the supernatant fraction by the Sigma Reagent Company's modification of the Reitman/Frankel procedure.

Urine 6-hr concentration test

The 6-hr concentration test used was that of Balaz, Hatch, Zawidska & Grice (1963). Drinking-water was removed from the cage, any urine was expelled from the bladders, and exactly 6 hr later urine was again expelled if possible. The animals were then placed in a metabolism cage equipped with a urine-faeces separator, and the specific gravity of the urine voided in the next hour was determined by micropyknometry. If no urine was obtained during this period the experiment was repeated later.

Serum chemistry

Blood samples were removed by cardiac puncture with the animals under light ether anaesthesia, and serum urea nitrogen and serum creatinine were determined with the Autoanalyser.

Terminal studies

At the end of the dosing period the animals were killed by bleeding under ether anaesthesia. The kidneys were weighed to the nearest milligram, fixed in 10% buffered formal saline and later prepared for histopathological evaluation.

RESULTS

Sixteen animals received phenacetin, sixteen received phenazone and twelve amidopyrine on 7 days a week according to the dose schedule laid out in Table 1. Sixteen control animals received vehicle alone.

TABLE 1
DAILY DOSE SCHEDULE OF ANALGESICS ADMINISTERED TO RATS

Day	Test I Phenacetin and phenazone (mg/kg)	Test II Amidopyrine (mg/kg)
1–6	750	500
7–14	850	750
15–20	1000	850
21–28	1100	950
29–36	1200	1100
37–51	1300	1200 (terminating on day 38)

Urine analyses were carried out frequently, on not less than four animals of each group throughout the test. On day 51 of Test I and day 35 of Test II all surviving animals had their serum urea nitrogen and creatinine determined.

The doses of the drugs administered were near the acute lethal level, so that during the test three of the phenazone, six of the phenacetin, and six of the aminopyrine-dosed animals died. Terminal studies were carried out on the remainder of the animals.

Cellular exfoliation

Figure 1 shows that the rate of cellular exfoliation was considerably raised in the phenazone-dosed animals compared with the controls and to animals given the other two drugs. This elevation in numbers of renal tubular cells was evident even on the second day of dosing and persisted throughout the test period. On six of the nine occasions when the urine was examined, the elevation in urinary cell count was significant [$P<0.05$] in the phenazone-treated animals compared with those receiving methyl cellulose alone. At no time was an elevation of renal cells demonstrable in the urine of phenacetin-treated animals. Amidopyrine gave a significant rise on only one occasion.

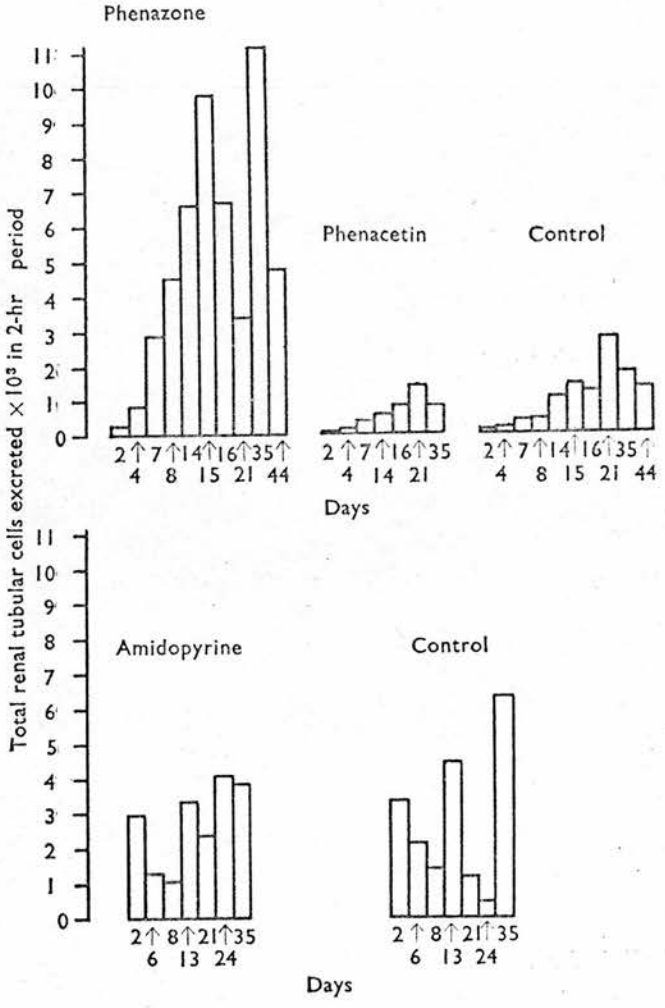

Fig. 1. Total renal tubular cells $\times 10^3$ excreted in a 2-hr period in the urine of not less than four rats treated orally with phenazone, phenacetin and amidopyrine according to the schedule in Table 1. The urine was obtained following an oral water load equivalent to 3% of their body weight.

Routine urine analysis

There was no obvious elevation of the urinary glutamic oxalacetic transaminase at any of the sampling dates, nor could the enzyme levels be correlated with cell count in the phenacetin and amidopyrine groups, but there was a marginal elevation in the phenazone group on several occasions. From the arbitrary score it was evident that there was also a significant increase in the incidence of albuminuria in this group, as is shown in Table 2. There was no evidence of any increased incidence of glycosuria.

TABLE 2

INCIDENCE OF ALBUMINURIA IN RATS DOSED WITH PHENACETIN, PHENAZONE AND AMIDOPYRINE

A score of ± is equivalent to less than 20 mg/100 ml., + 20–40 mg/100 ml., ++ 40–60 mg/100 ml. and +++ greater than 60 mg/100 ml. albumin

	Test I		
	Phenacetin	Phenazone	Controls
Total incidence of albuminuria	6 out of 32 animals 6+	24 out of 47 animals 12+ 11++ 1+++	8 out of 47 animals 8+

	Test II	
	Amidopyrine	Controls
Total incidence of albuminuria	4 out of 36 animals 1+ 2++ 1+++	4 out of 40 animals 3+ 1++

Six-hour urine concentration test

The results of the concentration test given in Table 3 show that with all three compounds there was a statistically significant depression of urinary specific gravity, the greatest effect occurring with phenazone and amidopyrine.

TABLE 3

6-HR URINE CONCENTRATION TESTS IN RATS

The specific gravity estimated by micro pycknometer at week 8 for the phenacetin- and phenazone-dosed rats and at week 5 for the amidopyrine dosed animals

	Test I		
	Phenacetin	Phenazone	Controls
Mean ± S.E.	1·0426 ±0·0038	1·0304 ±0·0039	1·0597 ±0·0052
t	2·65	4·53	
P	0·019	<0·001	

	Test II	
	Amidopyrine	Controls
Mean ± S.E.	1·0353 ±0·0038	1·0546 ±0·0041
t	3·36	
P	0·008	

Body weight and clinical condition

The terminal mean weights of the surviving animals are shown in Table 4. At the end of the 51 days in Test I with phenacetin and phenazone, and 38 days in Test II with amidopyrine, body weights of all the remaining test animals were depressed compared with the controls. The depression in body weight, which was significant in all instances, was more pronounced with amidopyrine than with the other two compounds.

TABLE 4

MEAN TERMINAL BODY WEIGHTS IN GRAMS OF RATS DOSED WITH PHENACETIN AND PHENAZONE (51 DAYS) AND AMIDOPYRINE (38 DAYS)

Numbers of animals are given in parentheses

	Test I		
	Phenacetin	Phenazone	Controls
Mean	376 (10)	361 (13)	428 (16)
t	2·70	4·23	
P	<0·05	<0·001	

	Test II	
	Amidopyrine	Controls
Mean	321 (6)	437 (12)
t	6·15	
P	<0·001	

Apart from the effect of body weight none of the animals showed any external signs, but serum urea nitrogen and serum creatinine levels were elevated in two out of six of the amidopyrine and one out of thirteen of the phenazone-dosed animals at the end of the test.

Organ weights

The mean gross kidney weight (Table 5) rose considerably in the amidopyrine-dosed animals. Table 5 shows the mean value compared with that for the other two analgesics and the controls.

TABLE 5

MEAN GROSS KIDNEY WEIGHTS IN GRAMS FROM SURVIVING RATS AT THE END OF THE TEST PERIODS. BOTH KIDNEYS WERE WEIGHED

Numbers of kidneys examined are given in parentheses

	Test I		
	Phenacetin	Phenazone	Controls
Mean	1·352 (20)	1·414 (26)	1·366 (32)
t	0·32	1·36	
P	>0·5	>0·2	

	Test II	
	Amidopyrine	Controls
Mean	1·896 (12)	1·350 (24)
t	4·01	
P	<0·001	

Histology

The most striking histological finding in paraffin sections of kidneys was in animals given amidopyrine. There was a sharply localized necrosis of the papillary tip in five out of six of these animals. This lesion was associated with a varying degree of tubular atrophy and dilatation, which in some cases occurred with an infiltration of cells of the chronic inflammatory type (Fig. 2). There was some congestion of the capillaries throughout the medulla, and occasional casts in the collecting tubules were also present in some animals.

In contrast, however, no papillary necrosis was seen in any of the phenacetin- or phenazone-treated animals. In one phenazone-treated animal there was much congestion throughout the entire tissue, with the occasional focus of cellular infiltration—again of the chronic inflammatory type—in the interstitial spaces.

Otherwise the kidneys from all other animals were normal.

Fig. 2. Photomicrograph (×125) of a haematoxylin and eosin stained paraffin section of the renal papilla of a rat treated orally with amidopyrine according to the dose schedule in Table 1. This shows a sharply localized necrosis in the papillary tip.

DISCUSSION

Most analgesics are commonly taken in the form of mixtures. The composition of these mixtures varies from country to country, but in broad terms in Europe the principal components are phenacetin and phenazone, or a near analogue such as amidopyrine, whereas in the United Kingdom and North America the analgesic constituents are usually aspirin and phenacetin. Phenazone and amidopyrine are not used to any extent in the Anglo-Saxon countries because of their recognized toxicity, particularly that of amidopyrine with its propensity to cause agranulocytosis.

In assessing the possible toxic effect of these mixtures it is virtually impossible to determine from human studies which compound is the one responsible and in the circumstances only animal studies carried out on the individual components can provide a satisfactory guide. Most studies in animals, however, have been inconclusive. A two-year study by Woodard, Post, Cockrell & Cronin (1965) in dogs with large doses of phenacetin failed to indicate any renal abnormality. In rats and rabbits, before a nephrotoxic action could be demonstrated it was necessary to infect the kidney with either *E. coli* (Miescher, Schnyder & Krech, 1958) or *Staphylococci* (Studer, Zbinden & Fust, 1958). On the other hand Abrahams, Rubenstein & Levin (1963) claim to have

induced papillary damage in rats to which tablets of phenacetin and also aspirin, phenacetin and caffeine were administered for 15 months. They, however, used Wistar rats, which have a high incidence of natural renal malformation, which could complicate the picture (Sellars, Rosenfeld & Friedman, 1960).

Animal experimentation, therefore, has so far provided little information about the effect of phenacetin on the kidneys and the circumstantial evidence points to the possibility of another agent being the cause.

Fazekas, Fazekas & Bertok (1960) studied experimentally in rabbits and cats the effect of high doses of amidopyrine, and have clearly demonstrated an effect on kidney function. Initially they found an oliguria, followed by a persistent polyuria, the animals ultimately dying in uraemia. Histological studies by the same authors showed that amidopyrine caused extensive necrotic changes in the kidney, particularly to the glomeruli and the vasa recti. The histological picture in these species, shown by Fazekas, Fazekas & Bertok (1962), does not, however, correspond to the classical picture of human pyelonephritis or interstitial nephritis as described by Thiel, Spuhler & Uehlinger (1964), but their results show unequivocally that amidopyrine causes serious damage to the kidney. On the other hand, the results of our experiments in rats have not only confirmed the nephrotoxic action of amidopyrine, but have also demonstrated that amidopyrine produces papillary necrosis similar to the chronic papillary necrosis in man (Knudsen, personal communication).

There are differences in the levels of exfoliation of renal tubular cells in the urine between the two groups of control animals, but phenazone caused a statistically significant elevation of cellular exfoliation in the urine over the control values, whereas amidopyrine, which caused extensive necrosis of the renal papillae, did not. Prescott (1956) demonstrated in man that mild analgesic drugs will cause an increase in the number of cells in the urine, aspirin having by far the greatest effect, whereas the effect of phenacetin was very much less, being only slightly greater than the control values. Taken in conjunction with Prescott's observations, our results indicate that the elevation of cells excreted in the urine need not necessarily suggest that the drug will cause actual renal damage. Nevertheless, phenazone and phenacetin have an effect on kidney function in rats as shown by a reduction in urinary concentrating ability, phenazone having a greater action in this respect than phenacetin. Kincaid-Smith (1967) has studied the pathogenesis of the renal lesion associated with the abuse of analgesics and has formed the view that renal papillary necrosis is the primary lesion. In view of the fact that amidopyrine and related drugs are used extensively in Europe, and from our own animal studies showing that amidopyrine will cause renal papillary necrosis, it would be of interest to carry out a retrospective epidemiological survey of the use of analgesic preparations to determine whether the incidence of nephritis associated with analgesic abuse is related to phenacetin or to the pyrazalone group of drugs.

SUMMARY

1. Phenacetin, phenazone and amidopyrine were given to rats in large doses by mouth over a period of 5 to 8 weeks and the effects of these drugs on kidney function were studied.

2. Phenazone causes a persistent celluria with evidence of slight kidney damage, whereas amidopyrine causes papillary necrosis but little if any celluria ; phenacetin causes neither.

3. The ability of the kidney to excrete a concentrated urine is adversely affected particularly by amidopyrine, to a lesser degree by phenazone, and to a very small extent by phenacetin.

4. It is possible that cases of "analgesic nephritis," reported from Europe, may be caused by the presence of amidopyrine and perhaps phenazone in most of the analgesic preparations used.

Our thanks are due to Dr. A. Knudsen, consultant histopathologist to the West Middlesex Hospital, and Dr. L. E. Mawdesley-Thomas, of Huntingdon Research Centre, for their interpretations of the histological specimens and to Professor C. A. Keele for helpful criticism.

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THE GANGLIONIC BLOCKING ACTIVITY OF A SERIES OF TERTIARY SULPHONIUM QUATERNARY AMMONIUM SALTS

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Received May 29, 1959

A number of polymethylene bis-tertiary sulphonium quaternary ammonium salts have been examined for their ganglionic blocking activity on the superior cervical ganglion of the cat. The effect of substituting different alkyl groups on the sulphur and nitrogen atoms and varying the length of the polymethylene chain has been investigated. Maximum activity was found in the tetramethylene di-ethyl sulphonium tri-ethyl ammonium salt, which was about three times as active as hexamethonium. With tri-, penta- and hexamethylene salts maximum activity occurs when there are a total of 13 to 15 carbon atoms in the molecule. It is concluded that the substitution of sulphur for nitrogen in the bis-quaternary ammonium salts does not necessarily lead to a reduction in relative potency. The importance of the groupings on the "onium" centres in producing ganglionic block is stressed.

The effect of substituting sulphur for nitrogen in polymethylene bis-quaternary ammonium salts has been investigated by several workers. Walker[1] has shown that replacing one nitrogen with sulphur in decamethonium led to a decrease in neuromuscular blocking activity. Muir and Lewis[2] confirmed this and demonstrated that the type of neuromuscular block produced by analogous sulphur-nitrogen compounds is unaffected. Barlow and Vane[3] synthesised a number of bis-alkyl tertiary sulphonium analogues of hexamethonium which proved to have less ganglionic blocking activity than their corresponding bis-quaternary ammonium salts. Wien[4] reported the replacement of nitrogen by sulphur in hexamethonium and its homologues to reduce activity. From these investigations it would appear that the replacement of nitrogen by sulphur in polymethylene bis-alkylammonium salts causes a decrease in relative activity.

We have investigated the ganglionic blocking activity of a series of straight chain polymethylene tertiary sulphonium quaternary ammonium salts synthesised by Doyle and Stove[5]. Among the compounds tested a number have been shown to possess ganglionic blocking activity in excess of hexamethonium.

Methods

The ganglionic blocking activity of compounds was assessed on the cat nictitating membrane preparation. The method was slightly modified from that described by Barlow and Vane[3]. Cats were anaesthetised with ether followed by intravenous chloralose-urethane mixture (0·7 per cent chloralose, 2·8 per cent urethane, 8·0 ml./kg.). A square wave stimulus of 1·0 millisecond duration and frequency of 14 shocks per second was

applied for 15 seconds every 3 minutes to the preganglionic fibres of the superior cervical ganglion. The voltage was adjusted to give just maximal contraction of the membrane. The nictitating membrane completely relaxed after each period of stimulation. The compounds were administered intravenously, into the femoral vein, 30 seconds before stimulation. The dose of hexamethonium bromide to give approximately 50 per cent inhibition of contraction (0·5 to 1·0 mg./kg.) was

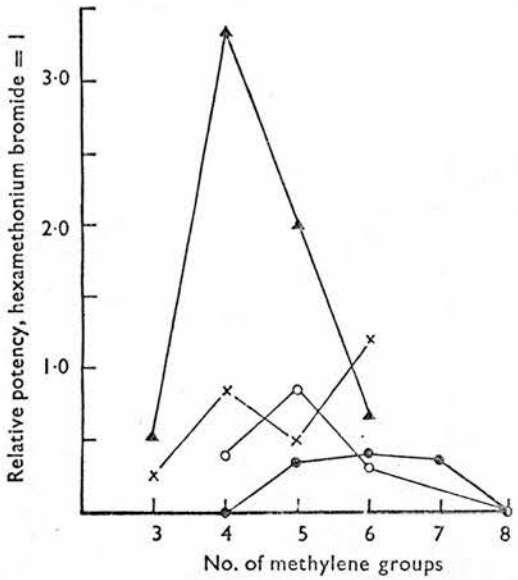

FIG. 1. Relationship between sympathetic ganglion blocking activity and chain length in a series of polymethylene bis-tertiary sulphonium quaternary ammonium salts.

General formula: $R_1R_2S^+(CH_2)_nN^+R_3R_4R_5$

	R_1	R_2	R_3	R_4	R_5
●—●	CH_3	CH_3	CH_3	CH_3	CH_3
○—○	CH_3	C_2H_5	CH_3	CH_3	C_2H_5
×—×	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5
▲—▲	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5

determined. Three doses of hexamethonium were then bracketed with two doses of the test compound and from the log-dose response lines obtained the relative potency of the compound was calculated.

The acute intravenous toxicities were determined in male mice weighing 18 to 22 g. and expressed as the LD50 in mg./kg.

RESULTS

Figure 1 illustrates the alteration in ganglionic blocking activity as a result of increasing the length of the polymethylene chain and substituting successively ethyl groups for methyl groups on the sulphur and nitrogen atoms. Compounds with methyl groups only on the sulphur and nitrogen have demonstrable ganglionic blocking activity, but this is always less

than hexamethonium. The pentamethylene, hexamethylene and heptamethylene compounds are equipotent, and have a relative ganglionic blocking activity of about 0·4 times hexamethonium. The tetramethylene and octamethylene compounds are inactive. When an ethyl group is substituted for a methyl group on both the sulphur and nitrogen atoms the relative activity within the series increases; the tetramethylene derivative has an activity of 0·4 times hexamethonium, while maximum activity occurs with the pentamethylene derivative which has a potency of 0·85

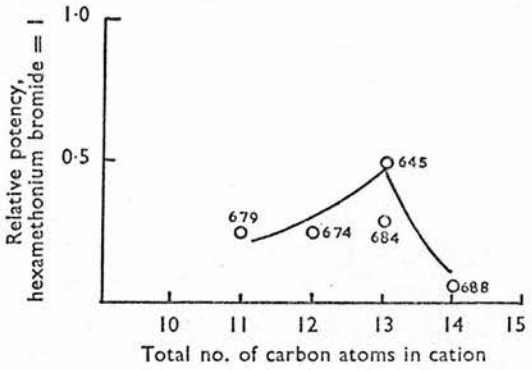

FIG. 2. Relationship between sympathetic ganglion blocking activity and total number of carbon atoms in the cation of a series of trimethylene bis-tertiary sulphonium quaternary ammonium salts.

General formula: $R_1R_2S^+(CH_2)_3N^+R_3R_4R_5$

Code No.	R_1	R_2	R_3	R_4	R_5
679	C_2H_5	CH_3	C_2H_5	C_2H_5	CH_3
645	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5
674	CH_3	CH_3	C_3H_7n	C_3H_7n	CH_3
684	CH_3	C_2H_5	C_3H_7n	C_3H_7n	CH_3
688	CH_3	C_3H_7n	C_3H_7n	C_3H_7n	CH_3

times hexamethonium. As the chain length is increased activity falls away rapidly and no significant ganglionic blocking activity is detectable in the octamethylene salt.

When a further methyl group is replaced by an ethyl group on the nitrogen atom, maximum activity occurs with the hexamethylene compound—the ethylmethylsulphonium di-ethylmethylammonium salt. This compound was slightly more active than hexamethonium. The higher members of this series, however, have not been investigated and therefore a complete picture of the variation in activity within the series has not been obtained.

Replacement of all the methyl groups by ethyl groups markedly alters the activity. There is a sharp increase in activity between the compounds having 3 or 4 carbon atoms in the chain. While the former is only 0·5 times as active as hexamethonium the latter is 3·3 times as active. The pentamethylene derivative is less active than the tetramethylene derivative, but is still twice as active as hexamethonium. The hexamethylene derivative has a relative potency of only 0·6.

TABLE I

SYMPATHETIC GANGLION BLOCKING ACTIVITY AND INTRAVENOUS TOXICITIES OF A SERIES OF $R_1R_2.S^+(CH_2)_4N^+R_3R_4R_5$ SALTS

Code number	Substituent groups					Toxicity in mice. LD50 mg./kg. I.V.	Ganglionic block hexamethonium bromide = 1·0
	R_1	R_2	R_3	R_4	R_5		
BRL 443	CH_3	CH_3	CH_3	CH_3	CH_3	194	<0·02
534	CH_3	C_2H_5	CH_3	CH_3	CH_3	170	0·2
519	CH_3	C_2H_5	CH_3	CH_3	C_2H_5	119	0·4
524	CH_3	CH_3	CH_3	C_2H_5	C_2H_5	103	0·4
644	C_2H_5	C_2H_5	CH_3	CH_3	C_2H_5	99	0·9
525	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5	54	0·9
653	CH_3	C_2H_5	C_2H_5	C_2H_5	C_2H_5	25	1·3
611	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	45	3·3
530	CH_3	CH_3	CH_3	C_3H_7n	C_3H_7n	75	0·6
683	CH_3	CH_3	CH_3	C_3H_7i	C_3H_7i	26	1·8
678	CH_3	C_2H_5	CH_3	C_3H_7i	C_3H_7i	26	2·5
654	CH_3	C_3H_7i	CH_3	C_3H_7i	C_3H_7i	20	0·4
531	CH_3	C_3H_7n	CH_3	C_3H_7n	C_3H_7n	40	0·2
652	CH_3	C_2H_5	C_2H_5	C_3H_7n	C_3H_7n	50	1·4
667	C_2H_5	C_3H_7n	C_2H_5	C_2H_5	C_3H_7n	31	0·4
589	CH_3	CH_3	CH_3	C_4H_9n	C_4H_9n	41	0·5

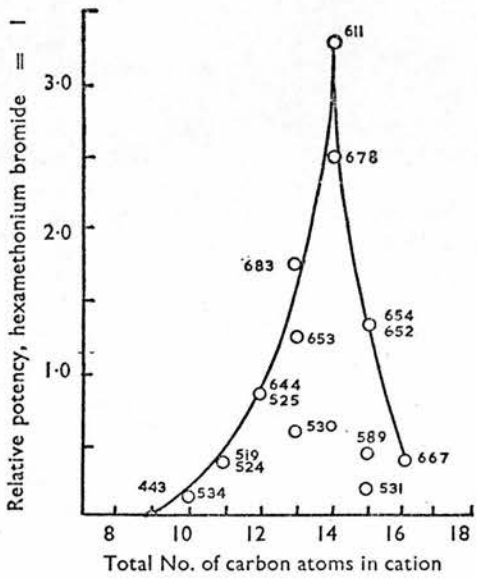

FIG. 3. Relationship between sympathetic ganglion blocking activity and total number of carbon atoms in the cation of a series of tetramethylene bis-tertiary sulphonium quaternary ammonium salts.

Numbers refer to structures shown in Table I.

The effect on the ganglionic blocking activity of substituting in turn methyl groups by larger groups in the tri-, tetra-, penta- and hexamethylene series was investigated further. The variation in activity of the compounds in the trimethylene series is illustrated in Figure 2. Maximum ganglionic blocking activity occurs when only ethyl groups are present on the sulphur and nitrogen atoms. When there are more than a total of 13 carbon atoms in the molecule activity is virtually abolished.

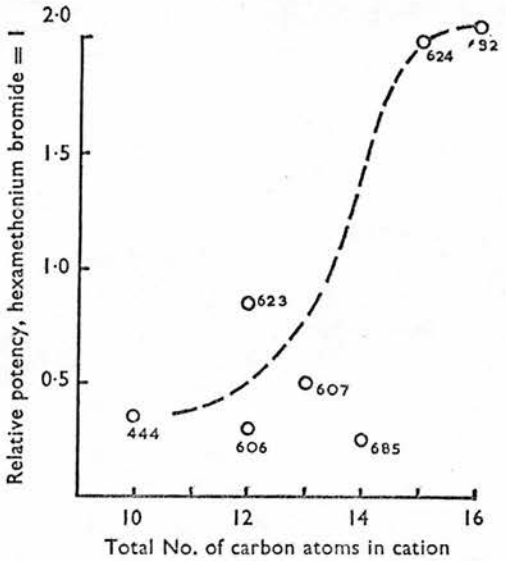

FIG. 4. Relationship between sympathetic ganglion blocking activity and total number of carbon atoms in the cation of a series of pentamethylene bis-tertiary sulphonium quaternary ammonium salts.

General formula: $R_1R_2S^+(CH_2)_5N^+R_3R_4R_5$

Code No.	R_1	R_2	R_3	R_4	R_5
444	CH_3	CH_3	CH_3	CH_3	CH_3
623	CH_3	C_2H_5	CH_3	CH_3	C_2H_5
606	CH_3	CH_3	CH_3	C_2H_5	C_2H_5
607	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5
624	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5
685	CH_3	CH_3	CH_3	C_3H_7n	C_3H_7n
682	CH_3	C_2H_5	C_2H_5	C_3H_7n	C_3H_7n

In the tetramethylene series a different picture evolves. The variations in the groupings and their relative ganglionic blocking activities obtained are given in Table I and are illustrated graphically in Figure 3. Maximum activity in this series again occurs when only ethyl groups are present on the sulphur and nitrogen atoms, but in this instance the compound is about 3 times as active as hexamethonium. Larger or smaller groups than ethyl on the sulphur and nitrogen atoms leads to diminished activity. There is also an interesting relationship between the compounds possessing *n*-propyl and isopropyl groups. A comparison between the dimethyl sulphonium di-isopropyl methyl ammonium salt (B.R.L. 683) and the

corresponding *n*-propyl isomer (B.R.L. 530) and a comparison between the methyl isopropyl sulphonium methyl di-isopropyl ammonium salt (B.R.L. 654) and the *n*-propyl isomer (B.R.L. 531) shows that the *n*-propyl compound is in both instances much less active than the corresponding isopropyl isomer.

The compounds prepared with the pentamethylene chain form a rather incomplete picture. The activities are expressed graphically in Figure 4. Maximum activity again occurs when only ethyl groups are present on the sulphur and nitrogen, but the ethylmethylsulphonium di-*n*-propyl ethyl

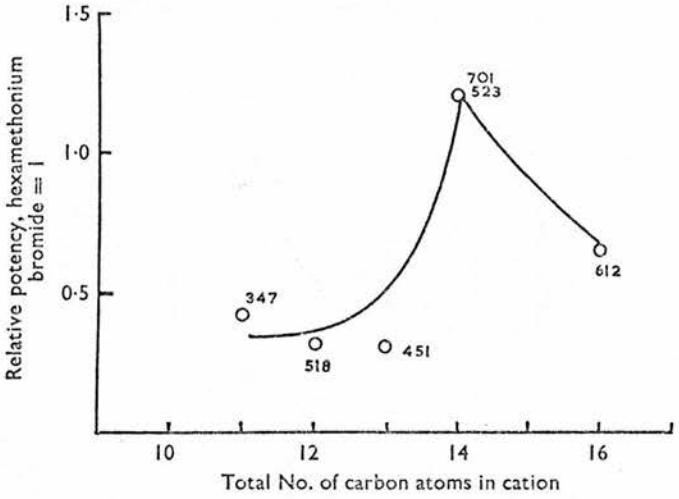

FIG. 5. Relationship between sympathetic ganglion blocking activity and the total number of carbon atoms in the cation of a series of hexamethylene bis-tertiary sulphonium quaternary ammonium salts. General formula: $R_1R_2S^+(CH_2)_6N^+R_3R_4R_5$.

Code No.	R_1	R_2	R_3	R_4	R_5
347	CH_3	CH_3	CH_3	CH_3	CH_3
518	CH_3	C_2H_5	CH_3	CH_3	CH_3
451	CH_3	C_2H_5	CH_3	CH_3	C_2H_5
701	C_2H_5	C_2H_5	CH_3	CH_3	C_2H_5
523	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5
612	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5

ammonium derivative is of the same order of activity. Compounds with larger groups than ethyl on the sulphur were not prepared, and hence it has been impossible to determine at which stage maximum activity does occur.

The ganglionic blocking activity in the hexamethylene series is illustrated in Figure 5. Maximum activity occurs with the diethyl sulphonium dimethylethyl ammonium salt, and the ethylmethyl sulphonium diethylmethyl ammonium salt. Both these compounds are slightly more active than hexamethonium. The diethyl sulphonium triethyl ammonium salt is about 0·6 times as active as hexamethonium. Compounds possessing larger groups on the sulphur and nitrogen were prepared, but none of these showed any significant ganglionic blocking activity.

Discussion

To achieve the maximum ganglionic blocking activity 4 carbon atoms are required in the carbon chain separating the sulphur and nitrogen atoms and the presence of only ethyl groups on the nitrogen and sulphur atoms is necessary. Barlow and Vane[3] similarly found that maximum activity in the hexamethylene bis-alkyl sulphonium series occurred when only ethyl groups were attached to the sulphur atoms. In the compounds with 3 carbon atoms in the chain the activity is not marked, but when the chain length is increased to 5 carbon atoms compounds with activity of twice that of hexamethonium have been obtained. Increasing the length of the chain to 6 carbon atoms likewise results in compounds with slightly greater activity than hexamethonium, but activity in this series follows a somewhat different pattern than seen in the preceding series. Relative activity would appear to follow more closely the change in activity seen with the bis-nitrogen compounds when the methyl groups are substituted in turn by ethyl groups as shown by Wien, Mason, Edge and Langston[6]. These workers found that the penta- and hexamethylene bis-dimethylethyl quaternary ammonium salts were the most active of the compounds tested, and of the 4 carbon chain compounds the bis-diethylmethyl ammonium salt was the most active. In the sulphur-nitrogen series the most active hexamethylene derivatives are the diethyl sulphonium dimethylethyl ammonium salt and the ethylmethylsulphonium diethylmethyl ammonium salts. Both are equiactive.

Wien and his colleagues also showed that the salt of hexamethylene bis-triethylammonium was practically devoid of ganglionic blocking action, but had a significant neuromuscular paralysing action. No such alteration in effect has been demonstrated in the sulphur-nitrogen series even with the substitution of larger groups. The shorter chain sulphur-nitrogen compounds would seem to be remarkably free from neuromuscular blocking action.

The modifications to structure of mono-quaternary and bis-quaternary ammonium salts which have shown ganglionic blocking activity has been investigated by several workers. Burn and Dale[7] found that substitution of the methyl groups by ethyl groups in tetramethylammonium abolished nicotinic stimulatory action on the blood pressure while maintaining the nicotine paralysing action. Subsequently Paton and Zaimis[8] demonstrated in a series of polymethylene bis-trimethylammonium salts the importance of the distance between the two onium centres. They showed that maximum activity occurred when the polymethylene chain contained 5 or 6 carbon atoms, the hexamethylene derivative being 20 times as active as tetraethylammonium. Wien and others[6] extended this work and investigated the effect of substitution of methyl by ethyl on the nitrogen atoms. They showed that optimal activity depended on both chain length and the nature of the substituents on the nitrogen atoms. The results of the present investigation emphasise the importance of the groupings on the onium centres and also stresses the fact that 5 to 6 carbon atoms separating the onium centres are not necessary for optimal activity. Nevertheless, depending on the type of onium centre present, an optimum

number of carbon atoms would appear to be necessary. In tertiary sulphonium quaternary ammonium compounds optimal activity occurs when there are only 4 carbon atoms in the polymethylene chain. However, to ensure an adequate fit on a receptor site, and so inducing ganglionic block, both the total length and general shape of the molecule appear to be of importance. That the volumes and effective radii of the groupings on the onium centre play a part in determining activity is evident from the difference in activity in the isomeric compounds containing *n*-propyl and *iso*propyl groups. The end groupings probably regulate the approach of the molecule to the receptor site and maximum ganglionic blocking activity is attained when there is correct electrolytic balance between drug and receptor site.

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Effect of Anti-inflammatory Agents on Capillary Permeability and Œdema Formation

FOLLOWING the injection of a noxious substance such as formalin into the subcutaneous tissues of an animal an inflammatory response ensues. If the injection is made into the feet (usually of rats), the degree of inflammation can be assessed by measuring the amount of swelling produced. The inflammatory response can also be measured by assessing the penetration of an intravenously administered dye into the affected area. The dye is bound to the plasma protein and therefore the amount of coloration produced gives an indication of the leakage of plasma protein through the capillary walls. Parratt and West[1] and Northover and Subramanian[2] have concluded from their experimental work that œdema formation and protein leakage are closely linked. On the other hand Gözsy and Kato[3] and Wilhelm[4] have indicated that processes are independent. In examining the action of anti-inflammatory drugs we have confirmed the views of the latter workers and have found that blueing and swelling can be affected independently.

The action of the drug on the blueing of mouse ears is assessed by giving intravenously a 0·5 per cent solution of 'Pontamine' sky blue in normal saline at a dose of 0·12 ml./10 g body-weight. The left ears of these mice are treated with xylol (2 drops to each surface of the ear applied with a syringe and needle) and in 15 min they become bright blue in the majority of mice. The reactors are picked out and randomly divided into groups (minimum number of 10 per group). Drugs at various dose-levels are given orally and 1 h later the right ear is treated with xylol. We have shown that in untreated control animals the blueing is unaffected between one ear and another under similar conditions. After 15 min the test is read as follows: if the blue of the left ear is more intense than the right ear a positive score is recorded. The percentage of positives is plotted against the dose on log paper and the ED_{50} value estimated graphically.

In the case of hydrocortisone, since the optimal time of testing would be expected to occur 4–6 h after oral administration, the test has to be modified as follows: 30 mice were taken and 15 were treated with hydrocortisone orally and the other 15 used as non-treated controls; after 4–6 h both groups of mice were treated with intravenous dye, then with xylol on the ears. After 15 min the ears were scored 0, 1, 2 and 3 according to the degree of blueness. Treated scores were compared with control scores by Student's *t* test.

Table 1. ANTI-INFLAMMATORY ED_{50} VALUES OF DRUGS BY VARIOUS TESTS (mg/kg orally)

Drug	Xylol	
	Blueing	Weighing
Aspirin	220	370
Chlorpromazine	2·5	5·4
Phenylbutazone	64	>400
Hexadimethrine bromide (polybrene (s.c.))	6·2	55
Hydrocortisone	ineffective	2·5

We also assessed the anti-inflammatory potency of the drugs by their effect on the increase in weight due to œdema of mouse ears when xylol is applied. Groups of 10 mice are treated orally with the drugs to be tested. A control group is also used. One hour later the right ears are treated with xylol as in the blueing test and after 30 min the mice are killed with chloroform and the ears cut off and weighed. The mean excess weight of the right ear is compared in control and treated groups, the effect of the drug being expressed as a percentage reduction of œdema formation, which is plotted against dose on log probit paper and the ED_{50} estimated. In the case of hydrocortisone, xylol was applied 4 h after treatment (this being the maximum time of effect).

The results obtained are summarized in Table 1. Hydrocortisone has little effect on the blueing reaction and is highly effective against the œdema formation. On the other hand, phenylbutazone and polybrene (hexadimethrine bromide) are relatively ineffective against œdema but active in preventing blueing. Aspirin and chlorpromazine have a dual action.

Armstrong and Stewart[5] have suggested that polybrene acts as an inhibitor of kinin formation *in vitro*, and since polybrene effectively inhibits the blueing reaction it would appear to have a similar action *in vivo*. Our experiments therefore are compatible with the view that kinins are liberated in the inflammatory response, but also show that œdema formation and protein transfer are two separate aspects of this response, kinin formation being associated with the latter aspect.

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